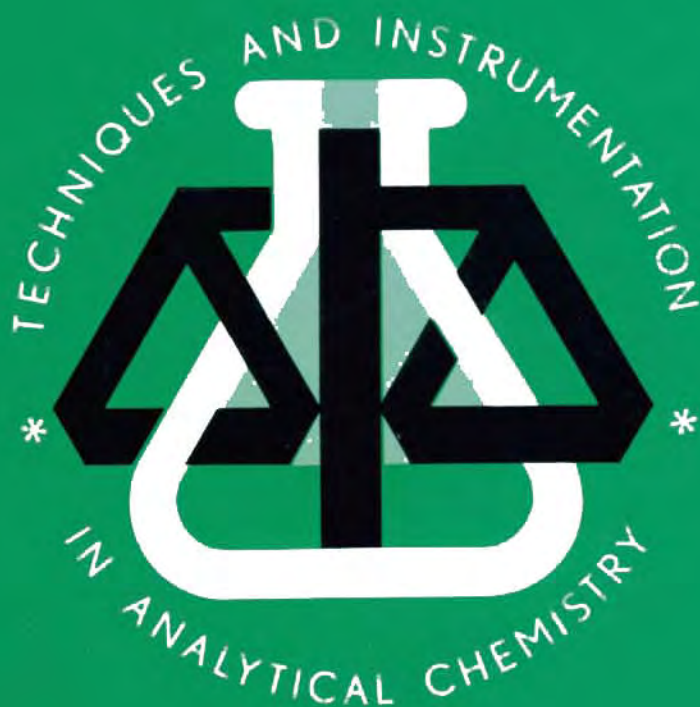


11



BIOSENSORS

Frieder Scheller and Florian Schubert

ELSEVIER

TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY — VOLUME 11

BIOSENSORS

TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY

- Volume 1 **Evaluation and Optimization of Laboratory Methods and Analytical Procedures. A Survey of Statistical and Mathematical Techniques**
by D.L. Massart, A. Dijkstra and L. Kaufman
- Volume 2 **Handbook of Laboratory Distillation**
by E. Krell
- Volume 3 **Pyrolysis Mass Spectrometry of Recent and Fossil Biomaterials. Compendium and Atlas**
by H.L.C. Meuzelaar, J. Haverkamp and F.D. Hileman
- Volume 4 **Evaluation of Analytical Methods in Biological Systems**
Part. A. Analysis of Biogenic Amines
edited by G.B. Baker and R.T. Coutts
Part B. Hazardous Metals in Human Toxicology
edited by A. Vercruysse
- Volume 5 **Atomic Absorption Spectrometry**
edited by J.E. Cantle
- Volume 6 **Analysis of Neuropeptides by Liquid Chromatography and Mass Spectrometry**
by D.M. Desiderio
- Volume 7 **Electroanalysis. Theory and Applications in Aqueous and Non-Aqueous Media and in Automated Chemical Control**
by E.A.M.F. Dahmen
- Volume 8 **Nuclear Analytical Techniques in Medicine**
edited by R. Cesareo
- Volume 9 **Automatic Methods of Analysis**
by M. Valcárcel and M.D. Luque de Castro
- Volume 10 **Flow Injection Analysis – A Practical Guide**
by B. Karlberg and G.E. Pacey
- Volume 11 **Biosensors**
by F. Scheller and F. Schubert

TECHNIQUES AND INSTRUMENTATION IN ANALYTICAL CHEMISTRY — VOLUME 11

BIOSENSORS

Frieder Scheller and Florian Schubert

*Akademie der Wissenschaften, Zentralinstitut für Molekularbiologie,
Robert-Rössle-Strasse 10, O-1115 Berlin-Buch, Germany*

in collaboration with

Ulla Wollenberger, Dorothea Pfeiffer,

Thomas Schulmeister, Reinhard Renneberg, Gerhard Etzold



ELSEVIER

Amsterdam – London – New York – Tokyo 1992

ELSEVIER SCIENCE PUBLISHERS B.V.
Sara Burgerhartstraat 25
P.O. Box 211, 1000 AE Amsterdam, The Netherlands

Distributors for the United States and Canada:

ELSEVIER SCIENCE PUBLISHING COMPANY INC.
655, Avenue of the Americas
New York, NY 10010, U.S.A.

ISBN 0-444-98783-5

© Akademie Verlag
Licensed edition for Elsevier Science Publishers B.V., 1992

This book is the revised translation of
Biosensoren
published by Akademie Verlag, 1989

All rights reserved. No part of this publication may be reproduced, stored in a retrieval system, or transmitted, in any form or by any means, electronic, mechanical, photocopying, recording or otherwise, without the prior written permission of the copyright owner.

No responsibility is assumed by the Publisher for any injury and/or damage to persons or property as a matter of products liability, negligence or otherwise, or from any use or operation of any methods, products, instructions or ideas contained in the material herein.

Although all advertising material is expected to conform to ethical (medical) standards, inclusion in this publication does not constitute a guarantee or endorsement of the quality or value of such product or of the claims made of it by its manufacturer.

This book is printed on acid-free paper.

Printed in The Netherlands

Contents

Preface	ix
Chapter 1. Introduction	1
Chapter 2. Physicochemical, Biochemical and Technological Fundamentals of Biosensors	7
2.1 Biosensors as Functional Analogs of Chemoreceptors	7
2.2 Structure and Function of Transducers	10
2.2.1 Thermometric Indication with Thermistors	10
2.2.2 Optoelectronic Sensors	13
2.2.3 Piezoelectric Sensors	18
2.2.4 Electrochemical Sensors	18
2.2.4.1 Potentiometric Electrodes	18
2.2.4.2 Amperometric Electrodes	24
2.2.4.3 Conductometric Measurement	34
2.3 Biochemical Fundamentals	34
2.3.1 Enzymes and Substrate Conversion	35
2.3.1.1 Structure and Catalytic Action	35
2.3.1.2 Classification of Enzymes	37
2.3.1.3 Enzyme Nomenclature	39
2.3.1.4 Components Affecting the Action of Enzymes	39
2.3.1.5 Kinetics of Enzyme-Catalyzed Reactions	41
2.3.1.6 Enzyme Activity and Enzyme Concentration	45
2.3.1.7 pH and Temperature Dependence	46
2.3.1.8 Inhibition of Enzyme Reactions	46
2.3.1.9 Isozymes and Allosteric Enzymes	46
2.3.2 Antibody-Antigen Interaction	48
2.3.3 Function of Receptors	49
2.4 Immobilization of the Receptor Component in Biosensors	50
2.4.1 Methods of Immobilization	51
2.4.1.1 Adsorption	51
2.4.1.2 Gel Entrapment	52
2.4.1.3 Covalent Coupling	52
2.4.1.4 Crosslinking	52
2.4.2 Immobilization Effects in Biosensors	53

2.4.3	Characterization of Immobilized Enzymes in Biosensors	56
2.4.3.1	Recovery of Enzyme Activity	56
2.4.3.2	Effectiveness Factor	57
2.4.3.3	Enzyme Loading Test	59
2.4.3.4	Concentration Dependence of the Signal and Linear Measuring Range	61
2.4.3.5	pH Dependence	64
2.4.3.6	Temperature Dependence	64
2.5	Mathematical Modeling of Amperometric Enzyme Electrodes	66
2.5.1	Fundamentals of Mathematical Modeling	66
2.5.2	The One-Layer Monoenzyme Electrode	70
2.5.3	One-Layer Bienzyme Electrodes	72
2.5.3.1	Enzyme Sequence Electrodes	73
2.5.3.2	Enzyme Competition Electrodes	75
2.5.3.3	Cyclic Enzyme Reactions	78
2.5.4	Multilayer Enzyme Electrodes	81
2.5.5	Conclusions	82
Chapter 3.	Metabolism Sensors	85
3.1	Monoenzyme Sensors	85
3.1.1	Glucose Sensors	85
3.1.1.1	Analytical Enzyme Reactors	89
3.1.1.2	Enzyme Membrane Sensors for Glucose	91
3.1.1.3	Enzyme-Chemically Modified Electrodes (ECME)	107
3.1.1.4	Biochemically Modified Electronic Devices	117
3.1.2	Galactose Sensors	125
3.1.3	Enzyme Electrodes for Gluconate	126
3.1.4	Lactate Sensors	126
3.1.5	Pyruvate Sensors	135
3.1.6	Determination of Alcohols	136
3.1.7	Sensors for Phenols and Amines	138
3.1.8	Cholesterol Sensors	144
3.1.9	Determination of Bile Acids	148
3.1.10	Determination of Glycolate, Glyoxylate and Hydroxybutyric Acid	149
3.1.11	Determination of Uric Acid	149
3.1.12	Determination of Ascorbic Acid (Vitamin C)	150
3.1.13	Determination of D-Isocitrate	152
3.1.14	Salicylate Sensors	153
3.1.15	Determination of Oxalate and Oxaloacetate	153
3.1.16	Determination of Nitrite and Nitrate	154
3.1.17	Sulfite Sensors	155

3.1.18	Determination of Carbon Monoxide	155
3.1.19	Electrochemical Sensor for Hydrogen Determination	156
3.1.20	Sensors for Amino Acids	157
3.1.21	Urea Sensors	159
3.1.21.1	Urease Reactors	161
3.1.21.2	Membrane Sensors	161
3.1.21.3	Biochemically Modified Devices	171
3.1.22	Creatinine Sensors	174
3.1.23	Penicillin Sensors	176
3.1.23.1	Enzyme Reactors	176
3.1.23.2	Membrane Sensors	178
3.1.23.3	Enzyme Field Effect Transistors	180
3.1.24	Determination of Glycerol and Triglycerides	181
3.1.25	Determination of Acetylcholine	182
3.1.26	Determination of Sucrose	183
3.2	Biosensors Using Coupled Enzyme Reactions	184
3.2.1	Enzyme Sequence Sensors	186
3.2.1.1	Enzyme Sequence Sensors for Disaccharides	187
3.2.1.2	Glucose Oxidase–Peroxidase (–Catalase) and Glucose Oxidase–Gluconolactonase Sensors	195
3.2.1.3	Glucose Isomerase–Glucose Oxidase Sensors	197
3.2.1.4	Sequence Electrodes for ATP and Glucose-6-phosphate	198
3.2.1.5	Enzyme Sequences Converting Lactate and Pyruvate	199
3.2.1.6	Cholesterol Oxidase-Cholesterol Esterase Sequence Sensors	204
3.2.1.7	Enzyme Sequence Sensors for Phosphatidylcholines and Acetylcholine	207
3.2.1.8	Multienzyme Electrodes for Creatinine and Creatine	209
3.2.1.9	Multienzyme Electrodes for Nucleic Acid Compounds, Phosphate and Fatty Acids	210
3.2.2	Competition Sensors	212
3.2.3	Enzymatic Elimination of Interferences	214
3.2.4	Substrate Recycling	220
3.3	Biosensors Using Higher Integrated Biocatalysts	230
3.3.1	Cell Organelles	232
3.3.2	Microorganisms	236
3.3.3	Tissue Slices	248
3.3.4	Miscellaneous Bioorganic Materials	251
3.3.5	Lipid Membrane Biosensors	252

Chapter 4. Affinity Biosensors	253
4.1 Affinity Sensors Using Low-Molecular Weight Ligands	253
4.2 Affinity Sensors Based on Proteins and Enzymes	254
4.2.1 Binding Sensors	254
4.2.2 Apoenzyme Electrodes for the Determination of Prosthetic Groups	259
4.2.3 Enzyme Sensors for Inhibitors	260
4.3 Immunosensors	264
4.3.1 Principles of Immunoassays	264
4.3.2 Electrode-Based Enzyme Immunoassays	266
4.3.3 Immunoreactors	271
4.3.3.1 Immunoreactors with Electrochemical Detection	271
4.3.3.2 Thermometric Enzyme Immunoassays	273
4.3.4 Membrane Immunosensors	275
4.3.5 Reagentless Immunolectrodes	280
4.3.6 Piezoelectric Systems	283
4.3.7 Optical Immunosensors	284
4.4 Biosensors Using Intact Biological Receptors	287
Chapter 5. Application of Biosensors	291
5.1 General Aspects	291
5.2 Biosensors for Clinical Chemistry	292
5.2.1 Test Strips and Optoelectronic Sensors	292
5.2.2 Thermistors	293
5.2.3 Enzyme Electrodes	293
5.2.3.1 Glucose	296
5.2.3.2 Urea	302
5.2.3.3 Lactate	304
5.2.3.4 Uric Acid	306
5.2.3.5 Determination of Enzyme Activities	307
5.3 Continuous Patient Monitoring and Implantable Sensors	311
5.3.1 Monitoring of Blood Glucose	311
5.3.2 Urea Determination in the Artificial Kidney	313
5.3.3 Determination of Lactate and Pyruvate	316
5.4 Food Analysis, Bioprocess Control and Environmental Monitoring	316
Chapter 6. Perspectives — Combination of Biotechnology and Microelectronics in Biosensors	323
List of Abbreviations and Symbols	327
References	329
Subject Index	353

Preface

Thousands of years ago man began to utilize biotechnological processes, for example in brewing, baking and wine-making. Having its roots in this tradition, and aided by the great advances of modern molecular biology, biotechnology has experienced an enormous growth in recent decades. Together with genetic engineering, novel immunotechniques, and protein engineering, biosensors nowadays appear at the leading edge of biotechnology in both research and practical applications. Biosensors are devices incorporating a biologically active element in intimate contact with a physico-chemical signal transducer and an electronic signal processor. The goal of this combination is to utilize the high sensitivity and selectivity of biological sensing for analytical purposes in various fields of research and technology.

This book is devoted in its entirety to a survey of present and some future aspects of biosensor research, development and application. The basic principles of the coupling of molecular recognition with signal processing in biosensors will be presented to an interdisciplinary readership in such a way as to achieve an understanding of the biochemical and technological peculiarities of that functional unit of micro electronics and biotechnology that is called a biosensor. Therefore, two main classes are distinguished according to the type of interaction leading to signal generation: metabolism sensors and affinity sensors. The technological aspect is stressed by discussing the sensors according to their inherent degree of integration.

After a short historical survey the fundamentals of signal transducers and the present state of thermometric, optoelectronic, and piezoelectric biosensors are presented. The most relevant electrochemical techniques are outlined in detail because electrochemical transducers are predominant. The aim of the second section is to provide information on the function of the biocomponents used in biosensors, primarily enzymes, but also antibodies and chemoreceptors. Special attention is paid to the methods of immobilization of the biomaterial and to the discussion and mathematical modeling of the interplay of biochemical reactions with mass transfer processes in immobilized enzyme electrodes.

The third chapter concentrates on metabolism sensors, which are arranged according to the degree of biocatalyst integration. The various different ways of coupling enzymes with transducers in monoenzyme sensors are exemplified by the determination of glucose and urea. The current state of the art is shown for monoenzyme sensors for some further 25 analytes and classes of analytes. Coupled enzyme reactions are shown to provide expansion of the biosensor concept to new analytes and to multiparameter assays as well as to an improvement of such analytical parameters as specificity and sensitivity. This chapter offers for the first time a complete overview of the potentials of coupled enzyme reactions in biosensors.

Biosensors using higher integrated biocatalytic phases, i.e. cell organelles, intact cells, and tissue material, are compared with isolated enzyme sensors. The merits of the former in the determination of 'complex variables', such as mutagenicity and nutrient content, are outlined.

Chapter 4 explores biosensors based on the generation of signals by binding events, i.e. affinity sensors. Sensors using low-molecular bio-specific ligands, non-enzyme proteins, apoenzymes, antibodies and antigens (immunosensors), and biological chemoreceptors are critically evaluated. The focus is on immunosensors which, on the one hand, rely on the universally applicable immunological reactions but, on the other hand, suffer from problems in the coupling of the signal transduction process.

Chapter 5 concerns the practical application of biosensors in clinical chemistry, patient monitoring, food analysis, process control and environmental monitoring. Various sensor types and commercial biosensor-based devices are compared on the basis of their analytical performance in 'real' samples.

The final chapter presents some exciting aspects of the future of biosensors, such as internal signal processing in the biocomponent of the sensor aiming at bioelectronic principles, and the possibility of tailoring the proteins for specific sensor configurations. The practical developments of these and other possible applications will require the concerted effort of researchers from many disciplines.

FRIEDER SCHELLER
FLORIAN SCHUBERT

Chapter 1

Introduction

A problem of paramount importance in analytical chemistry is selectivity, particularly at low analyte concentrations and in the presence of interfering substances. The sensitive and selective determination of a large number of compounds is of great relevance for scientific research as well as for several branches of industry, e.g., for process control in the chemical and food industries. In the field of health care it is indispensable for the diagnosis of diseases. Biotechnology, too, requires the analysis of complex media. High selectivity, even in trace analysis, has been gained by the considerable progress in analytical instrumentation, as is reflected by modern gas chromatography, high pressure liquid chromatography, mass spectrometry, and atomic absorption spectroscopy. However, owing to their high costs these powerful instrumental techniques are only used in specialized laboratories. Furthermore, they are in general not suited to on-line operation. The development of sensors which are highly selective and easy to handle is thus a key problem in analysis.

Whereas reliable sensors are available for the determination of *physical* parameters, e.g. temperature, pressure, or sound energy, the qualitative and quantitative analysis of *chemical* composition remains difficult. Electrochemical sensors, such as pH electrodes and Clark-type electrodes for oxygen measurement are widely used for this purpose. Ion selective electrodes and voltammetric sensors are particularly suitable for the determination of metal ions and various organic compounds. These electrochemical sensors, however, are scarcely applicable to the measurement of the majority of such physiologically important substances as glucose, urea or cholesterol. This is even more valid for biological macromolecules such as enzymes, antibodies or microorganisms.

Living beings are capable of recognizing and adapting to chemical

changes of their own metabolic state and their environment with high selectivity and sensitivity by using so-called receptors. The receptor systems consist of complex protein structures and are in most cases bound to cell membranes. They possess a high affinity for specific ligands which may be hormones, enzymes, or antibodies. Binding of the ligand

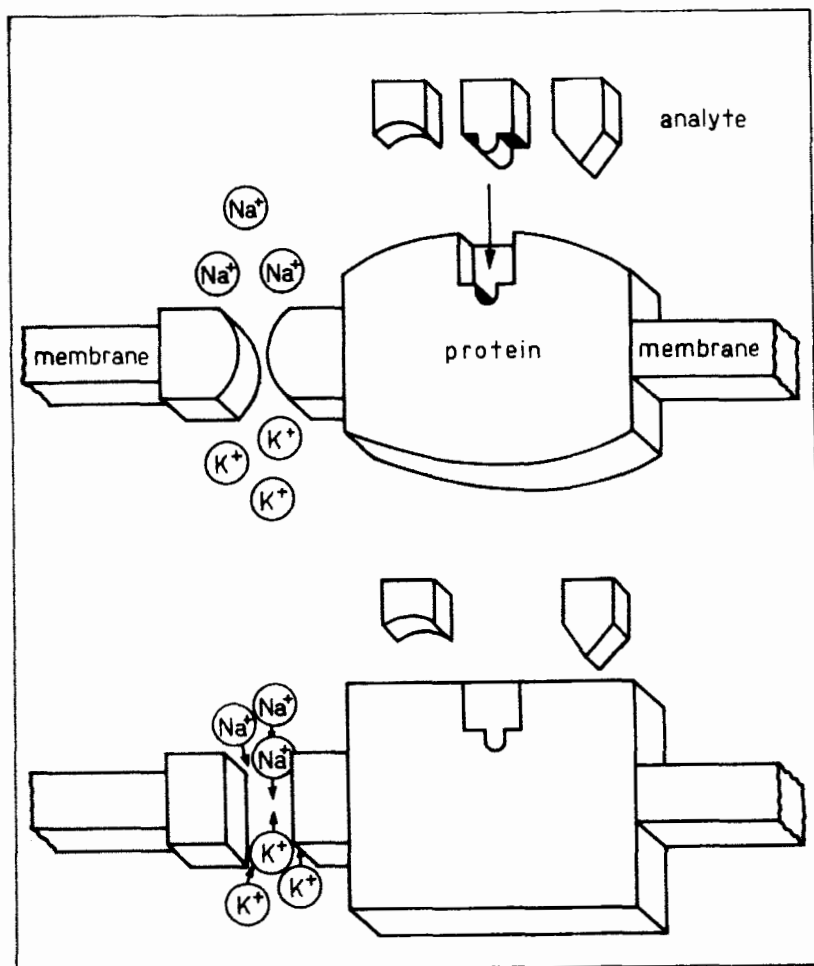


Fig. 1. Schematic of a natural chemoreceptor. The natural chemoreceptor possesses a specific site for binding of a substance, e.g. acetylcholine, and an ion channel traversing the membrane. The protein structure is changed as a result of substrate binding so as to allow for temporary opening of the channel and passage of sodium ions. The influx of sodium ions strongly changes the membrane potential. As little as a single binding event leads to a detectable signal.

causes activation of enzyme cascades via structural changes in the receptor protein. This results in a substantial amplification of the incoming signal (Fig. 1).

When the signal has been transmitted, the receptor–ligand complex is in most cases degraded inside the cell; the regeneration of the original state of the receptor therefore requires considerable time.

The outstanding specificity and the sensitivity of these biological receptors make them highly attractive for the development of sensors. However, owing to their structural complexity it appears to be extraordinarily difficult to use intact receptors in sensing devices. As a first step in this direction, enzymes — i.e. comparatively simple proteins — have been successfully applied in the molecular recognition of analytes.

Functioning as biological catalysts, enzymes specifically accelerate a huge number of chemical reactions at room temperature and normal pressure. The application of enzymes in analytical chemistry is not a new concept. As long as 55 years ago phosphatases were used as analytical tools. The importance of enzymes as ‘analytical reagents’ in clinical chemistry, food analysis and the pharmaceutical industry has been steadily increasing since that time. At present, enzymes are being routinely employed for the determination of about 80 different substances.

To simplify the enzymatic measurement of glucose, Free et al. (1956) adopted the principle of the litmus paper used for pH measurement. By impregnating filter paper with the glucose-converting enzymes they obtained the first ‘enzyme test strip’ which can be regarded as the predecessor of optoelectronic biosensors and which initiated the development and application of ‘dry chemistry’. Nowadays highly sophisticated enzyme test strips are commercially available for the determination of about 15 different low molecular weight metabolites as well as the activities of 10 enzymes.

Another route was chosen in the early 60s by L.C. Clark, the inventor of the Clark oxygen electrode (Clark and Lyons, 1962). Up to that time, for the electrochemical determination of glucose in blood samples several units of glucose oxidase (GOD) were added to the sample and the oxygen consumption resulting from glucose oxidation was followed with an oxygen electrode. Clark arranged the enzyme solution immediately in front of the O_2 electrode and avoided mixing the enzyme with the background solution by covering the reaction chamber with a semi-permeable membrane. Thus, a single enzyme preparation became

usable for several samples. This measuring arrangement introduced a new sensor concept — the biosensor. Biosensors are characterized by the direct spatial combination of a matrix-bound biologically active substance — the so-called receptor¹ — with an electronic device. For molecular recognition, biosensors may also be equipped with antibodies in place of enzymes. Besides nearly all kinds of electrodes various other signal transducers have been combined with the immobilized biomaterial.

The next stage was achieved in 1967 by Updike and Hicks, who entrapped GOD in a gel of polyacrylamide, thus increasing the operational stability of the enzyme and simplifying the sensor preparation. Further investigations by Reitnauer (1972) enabled the successful application of an enzyme electrode in a prototype blood glucose analyzer. In 1975 Yellow Springs Instrument Co. (USA) commercialized a glucose analyzer (model 23 A) which was based on a patent by Clark (1970). The 'Lactate Analyzer LA 640' by La Roche (Switzerland) followed one year later. In this instrument the enzyme is dissolved in a buffer in a reaction chamber placed in front of the electrode.

The development of biosensors is paralleled by that of analyzers using enzymes as dissolved reagents or immobilized in flow-through reactors coupled with electrochemical or spectrophotometric indication.

At the beginning of the 70s the first enzyme sensors with calorimetric indication (Mosbach, 1977) and later on those using optical indication, e.g., the light-emitting diode/photoreceptor system (Lowe et al., 1983), were developed. The extent of practical application of these measuring principles is far lower than that of enzyme electrodes.

Considering the high functional stability of enzymes in organelles and cells it appeared to be promising to employ these higher integrated biocatalytic materials in biosensors. As early as in 1975, Divies used bacteria in an alcohol sensor, whereas Guilbault constructed an NADH sensor by use of mitochondria in 1976. Application of highly stable immobilized enzyme models (synzymes) in biosensors (Ho and Rechnitz, 1987) might offer a new alternative to the employment of enzymes. Whilst the usefulness of enzymes is mainly restricted to the deter-

1 The term 'receptor' was introduced by Aizawa (1983) for that part of a biosensor which recognizes the analyte on the molecular level. In this broadened sense, it agrees with the definition by Scheler (1985). Recently Rechnitz (1987) introduced the term 'molecular recognition element' for the biocomponent of biosensors.

mination of low-molecular substrates or — via product indication — of enzyme activities, antibodies permit the measurement of macromolecular substances. Antibodies with high specificity are synthesized as part of the immune system of living organisms upon the introduction of foreign substances (antigens). The potential arsenal of 10^7 to 10^8 antibodies of different specificities in each animal is the source of the preparation of antibodies for a tremendous number of chemicals. They are widely used in immunoassays, which belong to the most successful achievements of bioanalytical chemistry of recent times. Several hundred millions of immunoassays are being conducted per year. The highly advanced immunotechniques suggested themselves as being fit for the biosensor concept. In 1975, Janata had already assembled a 'direct' immunoelectrode, and the electrochemical evaluation of enzyme immunoassays has now become common practice (Heineman and Halsall, 1985).

Apart from the application of complex biocatalytic systems intense efforts are being made to broaden the spectrum of measurable substances and to improve the analytical parameters of biosensors by the coupling of several different enzyme reactions. If the enzymatic conversion of the analyte does not result in a readily detectable physicochemical effect, further enzyme reactions can be coupled, leading to a measurable signal by conversion of the primary reaction product.

As early as 1970, Clark patented the sequential coupling of two enzymes, e.g., for the determination of disaccharides. Since this type of reaction is widespread in metabolism it can also be carried out by using cell organelles or microorganisms.

Other types of coupled reactions are those of parallel and cyclic substrate conversion. They, too, are 'copied' from nature. Optimization of these coupled reactions leads to systems approaching the functional parameters of biological receptors. On the other hand, the application of biological receptor proteins themselves for analytical purposes is being intensively studied. Thus, an affinity chromatography column with immobilized receptors has been devised (Ray et al., 1979) and, in 1986, Belli and Rechnitz described the first 'receptrode'.

A considerable body of research work is directed at the miniaturization of biosensors and the creation of multifunctional sensors by the use of small-scale electronic devices. Semiconductor biosensors have been developed by the biochemical modification of gas-sensitive or ion-sensi-

tive field effect transistors (FETs) (Danielsson et al., 1979). These enzyme, cell or immunotransistors may be regarded as the first examples of bioelectronics, being based on the combination of microelectronics and biotechnology (Karube and Morizumi, 1988).

Chapter 2

Physicochemical, Biochemical and Technological Fundamentals of Biosensors

2.1 BIOSENSORS AS FUNCTIONAL ANALOGS OF CHEMORECEPTORS

Biosensors are based on the direct spatial coupling of an immobilized biologically active compound with a signal transducer and an electronic amplifier (Fig. 2). They use biological systems at different levels of integration to specifically recognize the substance to be determined (Table 1). The first step of this interaction is the specific complex formation of the immobilized biologically active substance, R, with the analyte, S.

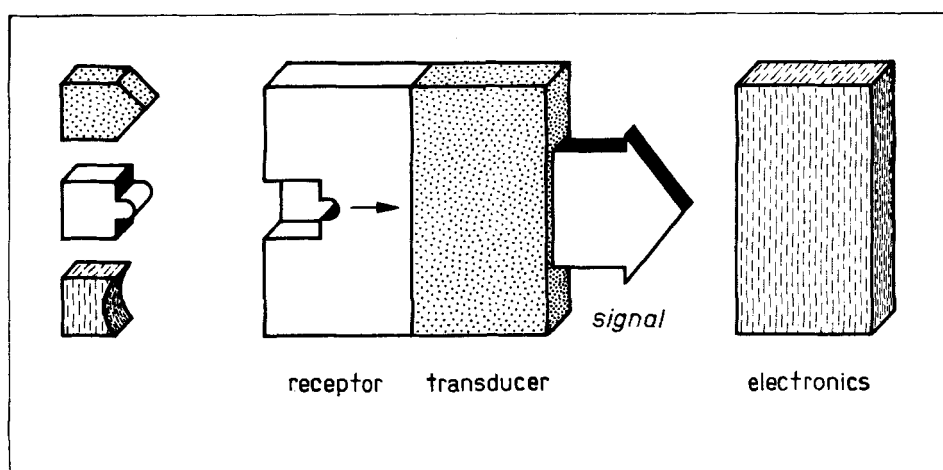


Fig. 2. Configuration of a biosensor.

TABLE 1

Principles of Biosensors

1. Bioaffinity sensors		2. Metabolism sensors	
$S + R \rightleftharpoons SR$		$S + R \rightleftharpoons SR \rightarrow P + R$	
change of electron density		substrate consumption and product formation	
<i>receptor R</i>	<i>chemical signal S</i>	<i>receptor R</i>	<i>chemical signal S</i>
dye	protein	enzyme	substrate
lectin	saccharide	organelle	cofactor
	glycoprotein	microbe	inhibitor
enzyme	substrate	tissue slice	activator
	inhibitor		enzyme activity
apoenzyme	prosthetic group		
antibody	antigen		
receptor	hormone		
transport system	substrate analogue		

Transducers

optoelectronic detectors, field effect transistors,
semiconductor electrodes, potentiometric electrodes,
amperometric electrodes, thermistors

3. Coupled and hybrid systems	4. Biomimetic sensors
sequence	<i>receptor R</i>
competition	carrier-enzyme
anti-interference	<i>physical signal S</i>
amplification	sound
	stress
	light

In analogy with affinity chromatography, in so-called *affinity sensors*¹, dyes, lectins, antibodies, or hormone receptors are being used in matrix-bound form for molecular recognition of enzymes, glycoproteins, antigens, and hormones. The physicochemical changes caused by the complex formation, e.g., change of layer thickness, refractive index, light

1 In the literature the two basic types of biosensors are also called binding sensors and catalytic sensors.

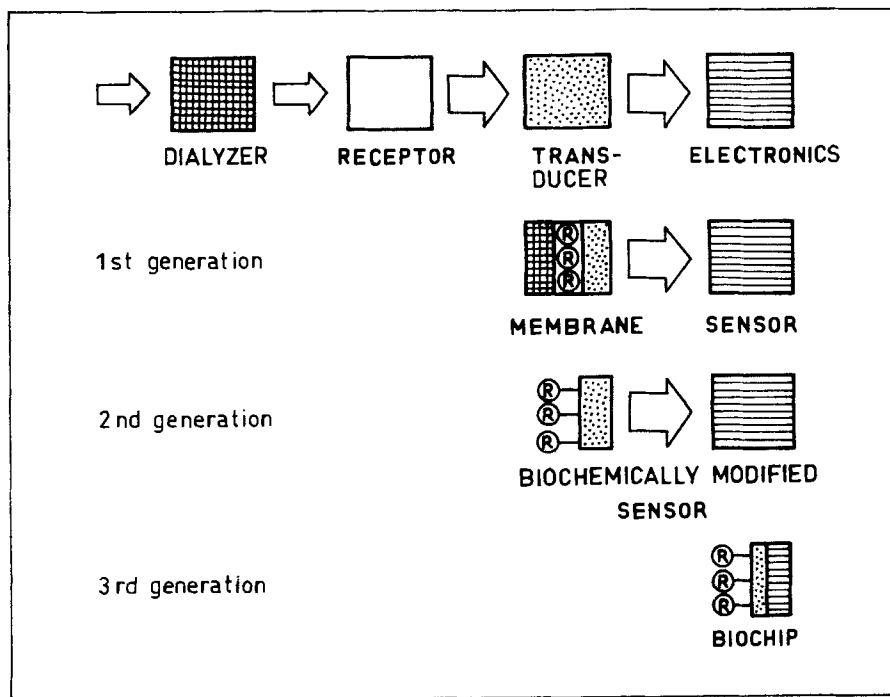


Fig. 3. Biosensor generations. R – receptor component.

absorption, or electrical charge, may be indicated by means of optoelectronic sensors, potentiometric electrodes, or field effect transistors. After the measurement the initial state must be regenerated by splitting of the complex.

On the other hand, the molecular recognition by enzymes, which are also applied in the form of organelles, microorganisms and tissue slices, is accompanied by chemical conversion of the analyte to the respective products. Therefore this type of sensor is termed a *metabolism sensor*². The initial state is usually reached when the analyte conversion is complete. With metabolism sensors, under certain conditions cosubstrates, effectors, and enzyme activities can be measured via substrate determination. Amperometric and potentiometric electrodes and thermistors are the preferred transducers, but in some cases optoelectronic sensors have also been used. With *biomimetic* sensors physical signals such as sound, stress, or light are measured through their ability to

² see previous footnote

influence the interaction between the biologically active material and its carrier. These sensors simulate the functions of the living senses.

In biosensors the following sequence of processes takes place:

- (i) specific recognition of the analyte;
- (ii) transduction of the physicochemical effect caused by the interaction with the receptor into an electrical signal;
- (iii) signal processing and amplification.

A similar sequence of events occurs in biological chemoreceptors under the action of very complex biomolecules and membranes.

According to their level of integration the biosensors described in the literature may be subdivided into three generations (Fig. 3). In the simplest approach (first generation) the biocatalyst is entrapped between or bound to membranes and this arrangement is fixed upon the surface of the transducer. The immediate adsorptive or covalent fixation of the biologically active component to the transducer's surface permits the elimination of the semipermeable membrane (second generation). The direct binding of the biocatalyst to an electronic device that transduces and amplifies the signal, e.g., the gate of a field effect transistor, is the basis for a further miniaturization of biosensors (third generation).

2.2 STRUCTURE AND FUNCTION OF TRANSDUCERS

The physicochemical change of the biologically active material resulting from the interaction with the analyte must be converted into an electrical output signal by an appropriate transducer. On the one hand, unspecific, but broadly applicable transducers may be used, which indicate general parameters such as reaction enthalpy (thermistor), mass change (piezoelectric crystal), or layer thickness (reflectometry). On the other hand, a specific indication may be achieved with potentiometric or amperometric electrodes for species such as H^+ , OH^- , CO_2 , NH_3 , or H_2O_2 , or with optical methods such as photometry or fluorimetry.

2.2.1 Thermometric Indication with Thermistors

Enzyme-catalyzed reactions exhibit the same enthalpy change as spontaneous chemical reactions, but inasmuch as they increase the reaction rate, also the rate of enthalpy change is substantially enhanced. Therefore thermometric indication is universally applicable in enzyme

sensors. Only one reaction step producing sufficient heat is required, and no 'measurable' reaction product must be formed. Table 2 shows the molar enthalpies of some enzyme-catalyzed reactions.

TABLE 2

Molar Enthalpies of Some Enzyme-Catalyzed Reactions

Enzyme	EC number	Substrate	$-\Delta H$ (kJ/mol)
Catalase	1.11.1.6	H ₂ O ₂	100.4
Cholesterol oxidase	1.1.3.6	cholesterol	52.9
Glucose oxidase	1.1.3.4	glucose	80.0
Hexokinase	2.7.1.1	glucose	27.6
Lactate dehydrogenase	1.1.1.27	pyruvate	62.1
β -Lactamase	3.5.2.6	penicillin G	67.0
Trypsin	3.4.21.4	benzoyl-L-arginine amide	27.8
Urease	3.5.1.5	urea	6.6
Uricase	1.7.3.3	uric acid	49.1

In preliminary investigations the enzyme was attached directly to a thermistor and this sensor was then dipped into the sample solution (Fig. 4) (Weaver et al., 1976; Schmidt et al., 1976). Difficulties caused by nonspecific thermal effects have been overcome by applying flow-through microcalorimeters. More recently, integrated-circuit temperature sensitive structures have been modified with enzymes. The differential output relative to an unmodified sensor is related to the temperature change induced by the enzyme-catalyzed reaction (Muramatsu et al., 1987a). Danielsson and Mosbach (1974) developed an enzyme thermistor where the temperature change in an enzyme reactor was registered by a thermistor device. Thus, enzyme reaction and indication have been spatially separated. Strictly, this means that enzyme thermistors are not truly biosensors according to the definition given above. In the literature, however, this setup is generally included in the biosensor area.

Enzyme thermistors contain a flow system in which the sample flows consecutively through an injector, the enzyme column and the temperature probe (Fig. 5). In most cases the sample flow is split, one part being pumped through an enzyme-free column with thermistor. To eliminate unspecific effects, the relevant signal is subtracted from that obtained

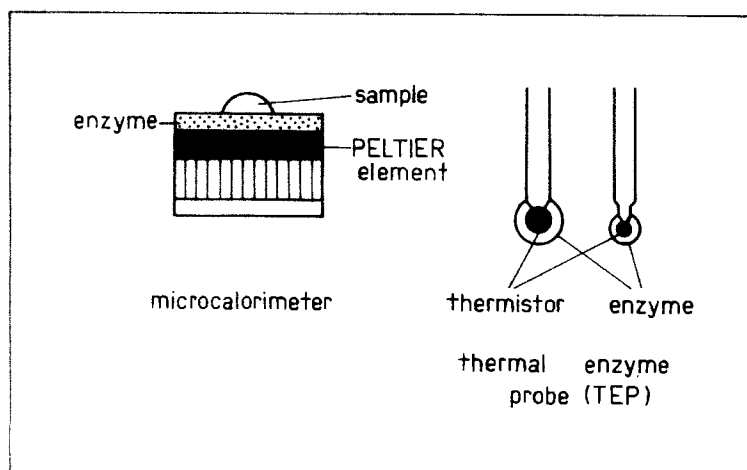


Fig. 4. Simple calorimetric biosensors. (Redrawn from Danielsson et al., 1981).

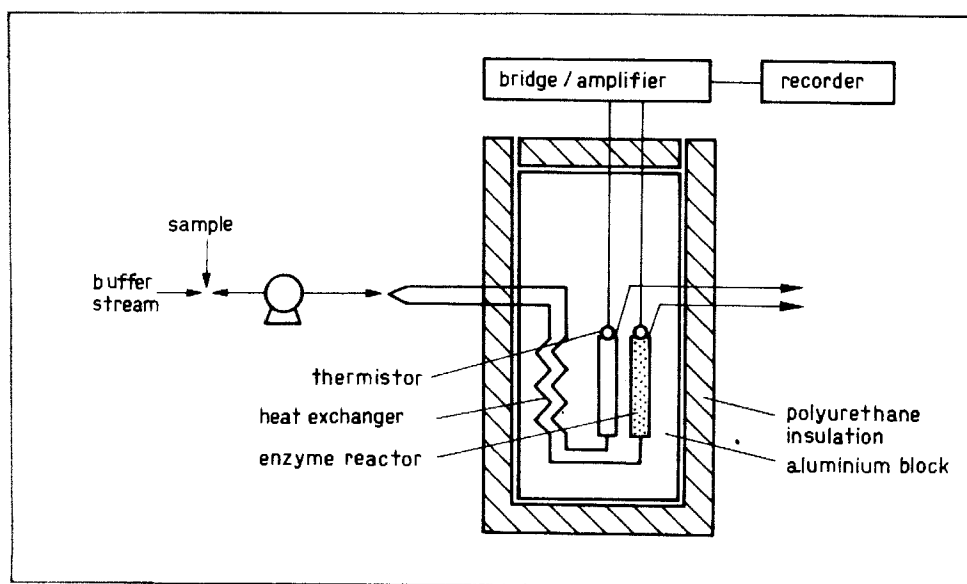


Fig. 5. Scheme of an enzyme thermistor. (Redrawn from Danielsson et al., 1981).

from the channel containing the enzyme column. The complete unit is contained in a thermostatically-controlled aluminum block of approx. 2 kg weight.

The magnitude of the measuring signal, ΔT , results from the change of enthalpy, ΔH , and the heat capacity of the device, c_P , according to:

$$\Delta T = n \Delta H / c_P,$$

n = number of moles of the analyte.

At the time of writing, more than 50 different analytes, such as substrates, enzymes, vitamins, and antigens, have been determined by the use of thermometric biosensors (Danielsson et al., 1981; Mattiasson and Danielsson, 1982; Danielsson and Mosbach, 1988). For substrate measurement the linear measuring range extends from about 0.1 mmol/l to 10 mmol/l; the sample frequency is about 10 per hour. In most cases, the enzyme is immobilized on porous glass and is stable for several weeks. Complete substrate conversion is achieved by immobilizing an excess amount of enzyme. The main advantages of the enzyme thermistor are its broadly universal applicability, its suitability for continuous measurement, and its independence of the optical properties of the sample. Because the devices are not easy to handle, however, their application has so far been restricted to some research laboratories and only one enzyme thermistor unit has reached a semi-commercial stage. A wider use may be expected.

2.2.2 Optoelectronic Sensors

The basic type of optoelectronic sensor combines light-conducting fibers with spectrophotometry, fluorimetry, or reflectometry. It is capable of indicating changes of optical parameters, such as light absorption, wavelength, or refraction index, in that part of the measuring medium immediately surrounding the fiber. These devices incorporate either a single or a dual optical fiber bundle for the incident light and for the light beam to be measured (Fig. 6). If only one fiber bundle is used it is necessary that the incoming and the indicated radiation differ either in time (pulsed light) or in wavelength (e.g., with fluorescence or luminescence). The incident light must be within the critical angle for total internal reflection; therefore the geometrical shape of the sensor surface is of essential importance (Fig. 7).

The most important field of application of this sensor type is the determination of cell fluorescence, which depends on the intracellular NADH/NAD⁺ ratio and is thus a sensitive measure of the cell state (Harris and Kell, 1985).

'Chemical' optoelectronic sensors use a reagent, R, which is immobi-

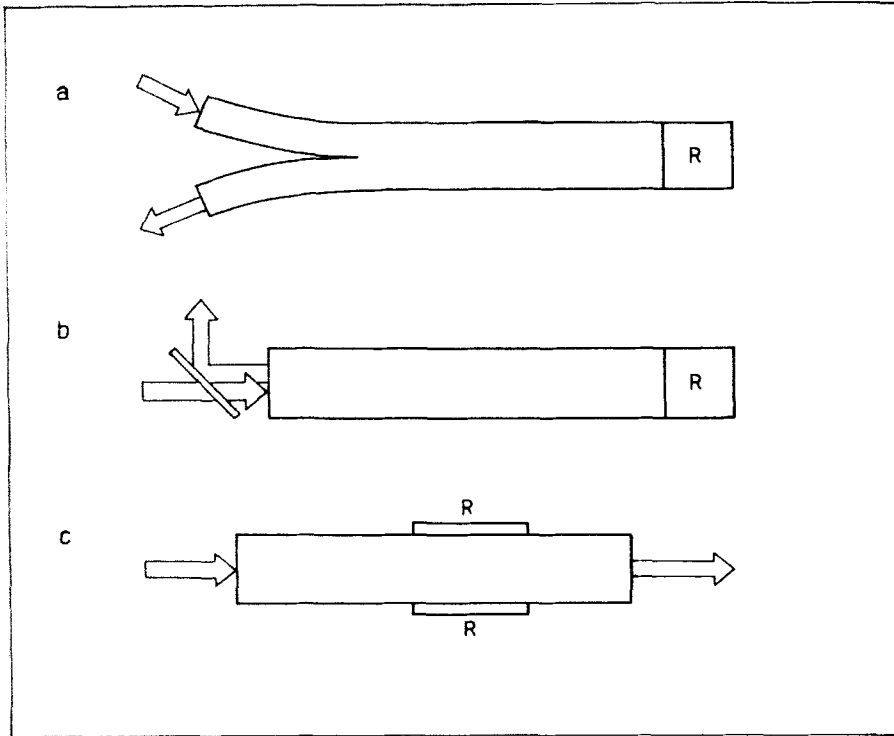


Fig. 6. Types of optoelectronic biosensors based on fiber optics. R – chemically sensitive reagent. (a) Bifurcated fiber optic sensor; (b) single fiber optic with a beam splitter for separation of incident and reflected light; (c) single fiber optic in which the reagent phase is coated on the optic.

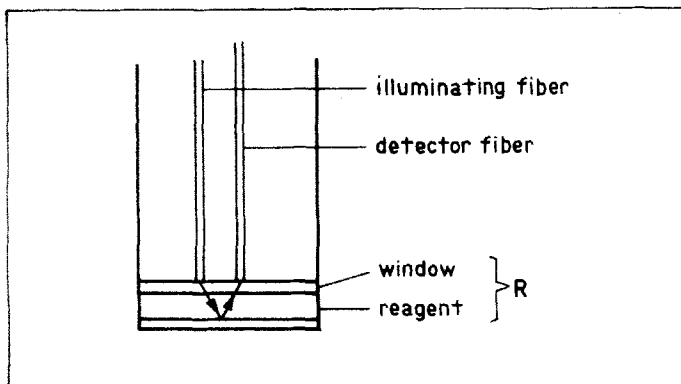


Fig. 7. Light transmission in optoelectronic fiber-based sensors.

lized at the sensitive surface (most often the tip of the fiber) and changes its optical properties in dependence on the composition of the measuring solution (Fig. 6). For pH measurement various fluorescence dyes, and for ammonia measurement oxazine perchlorate are currently being used to modify optical sensors (Seitz, 1984; Wolfbeis, 1989). Oxygen may be indicated by fluorescence quenching of aromatic compounds (Wolfbeis, 1987; Opitz and Lübbers, 1987).

Optical sensors offer a number of advantages: they are not susceptible to disturbances by electric fields, they are suited for continuous indication, and, during the measurement the sample remains chemically unchanged. On the other hand, they may be operated only in the dark, since daylight disturbs the measuring procedure.

Optical biosensors comprise a rather heterogeneous group of sensors in which the interaction of light with an immobilized biologically active material is sensed. They often contain a light source in addition to the signal transducer.

The determination of nitrophenyl phosphate using alkaline phosphatase (Arnold, 1985) has been used as a model system for analytically important substrates. The enzyme is entrapped in the reagent layer of the optical sensor depicted in Fig. 7. The nitrophenyl phosphate substrate permeates from the sample into the layer and is there converted to the strongly yellow nitrophenol. The change in light absorption is proportional to the substrate concentration.

Membrane-covered optochemical sensors (optodes) with O_2 sensitive or pH sensitive fluorescence indicators (e.g. pyrene butyric acid or hydroxypyrene trisulfonic acid) have been coupled with different enzyme reactions, such as the conversion of glucose, lactate, ethanol, or xanthine, and with antigen-antibody couples (Opitz and Lübbers, 1987).

Colorimetric and fluorimetric NH_3 sensors contain mixtures of pH indicators having suitable dissociation constants at the tip of the fiber bundle. The measuring solution is separated from this indicator layer by an NH_3 gas-permeable membrane covered by an immobilized deaminating enzyme, e.g. urease (Wolfbeis, 1987; Arnold, 1987). The fluorimetric indication of NADH has been used in optical biosensors for lactate, pyruvate, and ethanol, where the respective dehydrogenase is immobilized at the tip of an optical NADH sensor (Arnold, 1987; Wangsa and Arnold, 1988).

Common spectrophotometers, which contain the sample between a light source and a light detector, are used in optical biosensor systems, too. Figure 8 shows a pH indicator coimmobilized with enzyme, e.g.

glucose oxidase, urease, or β -lactamase, on a cellulose membrane sensing the colour change upon conversion of the respective analyte: glucose, urea, or penicillin (Lowe et al., 1983; Lowe and Goldfinch, 1988). Optical fiber sensors with immobilized oxidases for the determination of glucose, uric acid and cholesterol have been developed by Kobayashi et al. (1981). Here, the chemiluminescence of the H_2O_2 -dependent conversion of luminol is used as the measuring signal. The indication of light emitted at the surface of a light-conducting fiber bundle under the action of peroxidase or luciferase permits the measurement of hydrogen peroxide, ATP, and NADH (Freeman and Seitz, 1970). In an affinity sensor for glucose based on concanavalin A the fluorescence alteration of fluorescein-labeled dextran is indicated (Schultz and Sims, 1979).

In Fig. 8 a sensor for the determination of serum albumin is shown. The color change due to the formation of the complex with bromocresol green is used as the measuring signal.

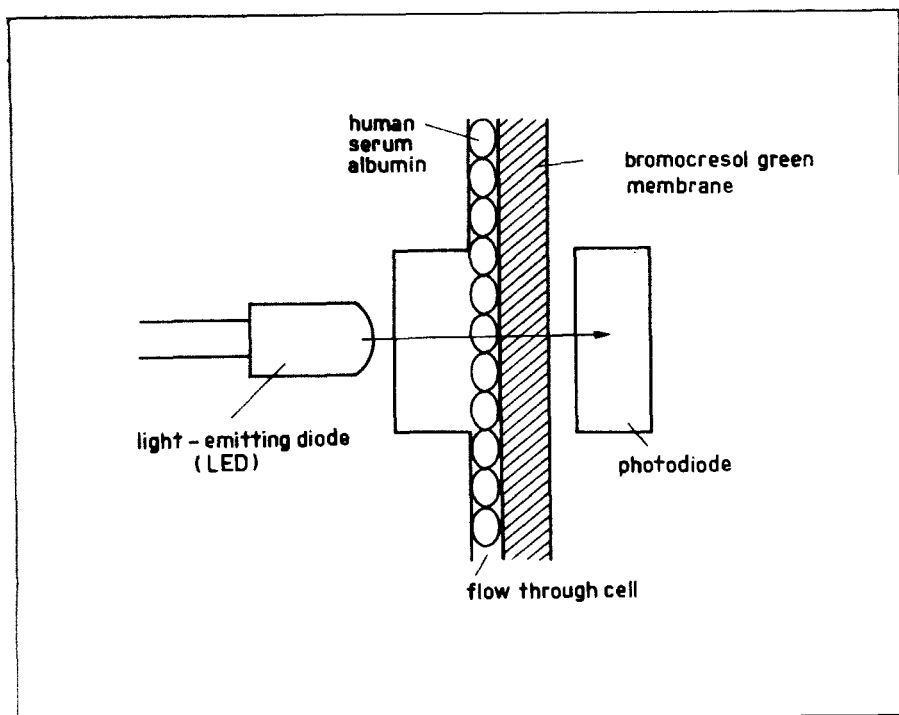


Fig. 8. Schematic of an optoelectronic sensor for the determination of human serum albumin by using immobilized bromocresol green. (Redrawn from Lowe et al., 1983).

The indication of changes of light polarization by ellipsometry or of changes in layer thickness by reflectometry allows the determination of macromolecules without the coupling of auxiliary reactions (Elwing and Stenberg, 1981; Place et al., 1985). In this way the complex formation between high-molecular antigens and antibodies adsorbed on a reflecting silica surface may be sensed directly (Fig. 9).

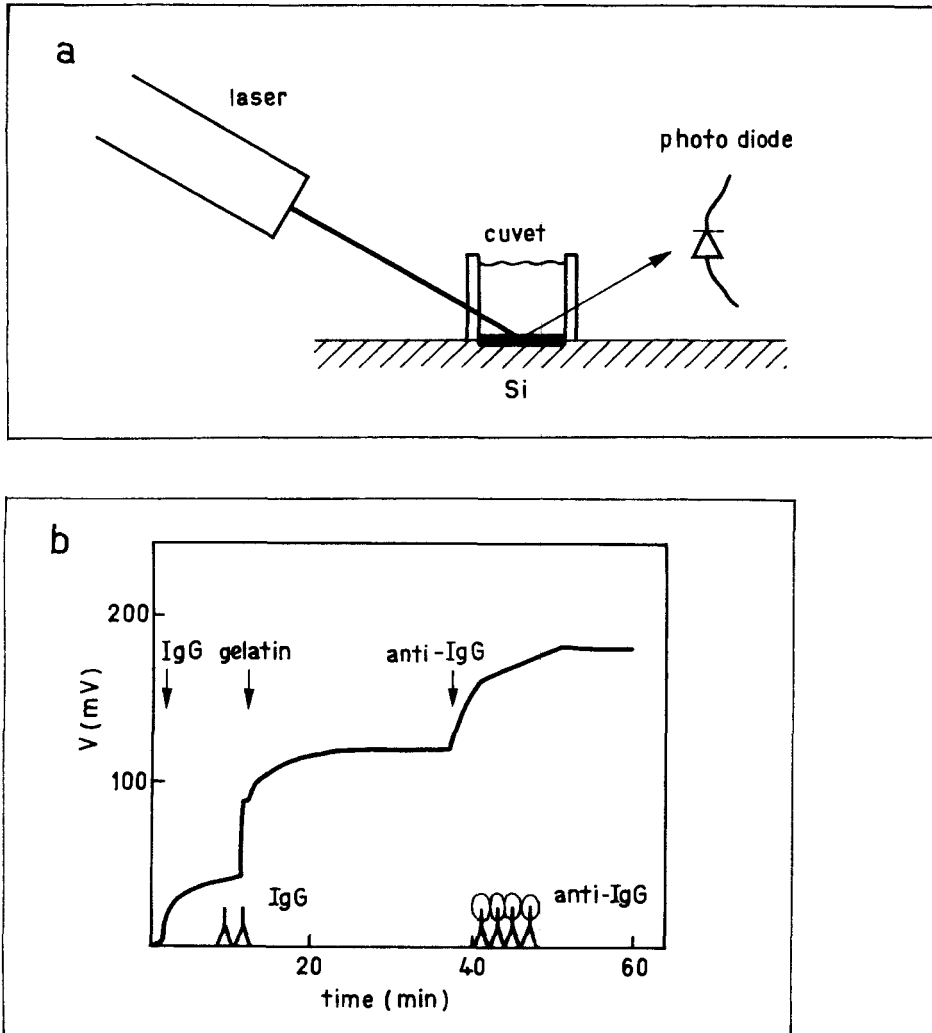


Fig. 9. Reflectometric immunosensor. (a) Construction scheme; (b) dependence of the measuring signal on layer thickness upon reaction between adsorbed immunoglobulin G (IgG) and anti-IgG. (Gelatin is added to suppress nonspecific adsorption on the free silicon surface.) (Redrawn from Welin et al., 1984).

Optoelectronic biosensors are advantageous in that they require no reference signal and no electrical shielding. Furthermore, the sensitivity of the optode is readily adaptable to the desired measuring range.

2.2.3 Piezoelectric Sensors

The principle of this sensor type is that the frequency of vibration of an oscillating crystal is decreased by the adsorption of a foreign material on its surface. The crystal is sensitized by covering it with material binding or reacting with the analyte. Piezoelectric sensors are used for the measurement of ammonia, nitrous oxides (NO_x), carbon monoxide, hydrocarbons, hydrogen, methane, sulfur dioxide, and certain organophosphate compounds (Guilbault, 1980). Adaptation of this measuring technique to aqueous systems, i.e. to the conditions common for biological systems, is difficult. A successful application has been described by Mandenius and Guilbault (1983), who covered a piezoelectric crystal directly with an immobilized enzyme. Piezoelectric immunosensors have also been proposed (Shons et al., 1972; Guilbault and Ngeh-Ngwainbi, 1987).

2.2.4 Electrochemical Sensors

Clearly, electrochemical indication prevails over all other methods of transduction. Potentiometric and amperometric enzyme electrodes are at the leading edge of biosensor technology with respect to the body of scientific literature as well as to commercially available devices (Schindler and Schindler, 1983). Only a few conductometric biosensors have been described, but the relevance of this sensor type may increase because of the relative ease of their preparation and use. Furthermore, the development of biochemically sensitized field effect transistors, being at present only at an initial stage, offers new prospects (Pinkerton and Lawson, 1982).

2.2.4.1 Potentiometric Electrodes

The simplest potentiometric technique is based on the concentration dependence of the potential, E , at reversible redox electrodes according to the Nernst equation:

$$E = E_0 + \frac{RT}{nF} \ln a_S.$$

where E_0 = standard redox potential, R = gas constant, T = absolute temperature, F = Faraday constant, n = number of exchanged electrons of the substance S, and a_S = activity of the substance S.

Examples of this measuring principle are the ferricyanide/ferrocyanide and benzoquinone/hydroquinone redox systems. In contrast, application of this electrode type to the system O_2/H_2O_2 contradicts the electrochemical preconditions.

In *ion selective electrodes* (ISE) the dissociation of groups contained in a sensitive membrane causes alterations of the charge density and thus changes of the membrane potential (Fig. 10). The activity and the potential are logarithmically related. To account for the influence of disturbing ions, e.g., of a substance P, a selectivity coefficient, $k_{S,P}$ is introduced:

$$E = \text{const} + \frac{RT}{Z_S} \ln (a_S + k_{S,P} (a_P)^{Z_S/Z_P})$$

where Z represents the charge of the ion.

The most important ion selective electrode using a 'solid membrane' is the glass electrode for pH measurement. Despite their outstanding selectivity for H^+ ions glass electrodes are used only seldom in enzyme electrodes because their sensitivity is affected by the buffer capacity of the measuring solution.

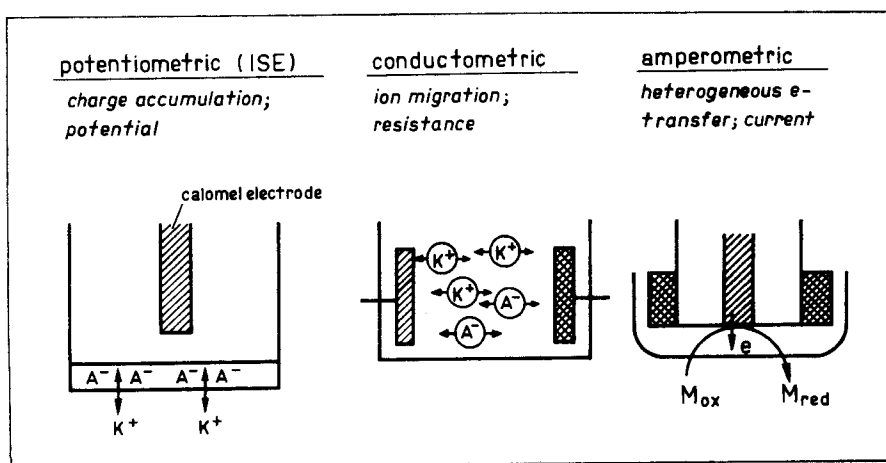


Fig. 10. Electrochemical transducers of biosensors. A^- : anion, K^+ : cation, M_{ox} : oxidized mediator, M_{red} : reduced mediator.

Liquid membrane ISEs are based on ion exchangers or neutral carriers included in a thin layer of water-immiscible solvent. In most cases the phase boundary is stabilized by a porous carrier membrane. Hydrophobic compounds having a high binding constant for the ion to be determined act as ion exchangers. The antibiotics nonactin and valinomycin serve as neutral carriers for the determination of NH_4^+ and K^+ , respectively (Štefanac and Simon, 1966; Simon, 1987).

The selectivity of the glass electrode for NH_3 and CO_2 may be improved over that of pH measurement by inclusion of a gas permeable membrane of about 20 μm thickness (e.g., of polyethylene or polytetrafluoroethylene) between the measuring solution and a flat pH electrode. In this way a gas sensitive ISE is formed (Stow and Randall, 1973). Under equilibrium conditions the partial pressure of the permeating gas in the measuring solution corresponds to that in the electrode-contacting electrolyte layer. Therefore, at constant solution pH a defined relation exists between the potential of the glass electrode and the concentration of the gas-forming ions HCO_3^- or NH_4^+ . Maximum sensitivity of the electrode is reached when the H^+ concentration in the solution is sufficient to ensure maximum conversion of the weak electrolyte into its undissociated form, i.e. CO_2 or NH_3 . With NH_3 this occurs at $\text{pH} > 10$, with CO_2 at $\text{pH} > 5$. Generally these pH values differ substantially from the pH optima of deaminase and decarboxylase enzymes; therefore, for the respective enzyme electrodes a compromise pH has to be found. To obtain optimal conditions for both steps, the enzyme reaction is often separated from the potentiometric indication and a pH change is included between these stages. This setup is termed a *reactor electrode*.

The semipermeable membrane may be replaced by an air gap between the measuring solution and the pH electrode (Ruzicka and Hansen, 1974). This increases the measuring rate but affects the electrolyte layer and thus the reproducibility of the measurement.

Other pH-sensing transducers used in biosensors are metal oxide electrodes. Beside the common antimony oxide electrode, palladium oxide and iridium oxide probes have been coupled with immobilized enzymes. These sensors may be miniaturized by using chemical vapor deposition technology. Moreover, they are mechanically more stable than glass electrodes. Unfortunately the measuring signal of metal oxide electrodes is affected by redox active substances.

Ion sensitive field effect transistors (ISFETs) have been directly derived from ion sensitive electrodes. To eliminate electrical disturbances caused by the high resistivity of the sensor the impedance

transformer has been integrated into the electrode body. As a further step, the ion sensitive membrane has been fixed directly to the field effect transistor (Bergveld, 1970; Janata and Moss, 1976). In this way, both the integration of electronic signal processing and the miniaturization of the sensor can be realized. For the construction of 'microbiosensors', pH sensitive ISFETs (Fig. 11) have been coupled with enzyme reactions (Van der Schoot and Bergveld, 1987/1988).

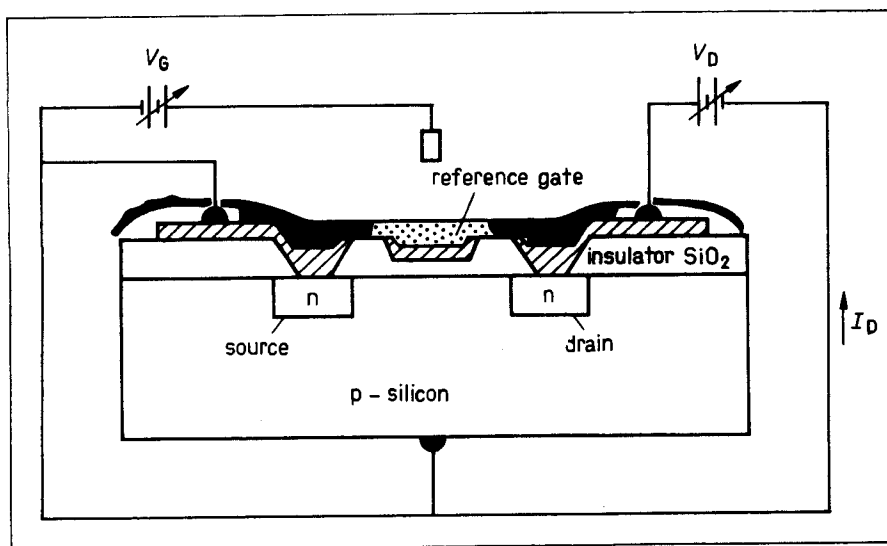


Fig. 11. Construction of an ion sensitive field effect transistor. The drain current (I_D) is determined by the gate potential (V_G) and the potential difference (V_D) between gate and source.

A potentiometric silicon-based sensor which indicates the local pH or redox potential at the surface has been devised by Molecular Devices (USA) (Hafeman et al., 1988). The photocurrent generated by illumination of the back side, containing an array of light-emitting diodes, reflects the bias potential across the sensor. In this manner, changes of the surface potential generated by enzyme reactions can be detected. For high-sensitivity measurements the enzyme reaction is conducted in a reaction chamber of only 1 nl volume; thus, as few as 10 000 enzyme molecules are required to produce a measurable response within 1 minute. Therefore, a broad spectrum of analytes, including enzymes and enzyme-linked assays (enzyme immunoassays and DNA probe assays),

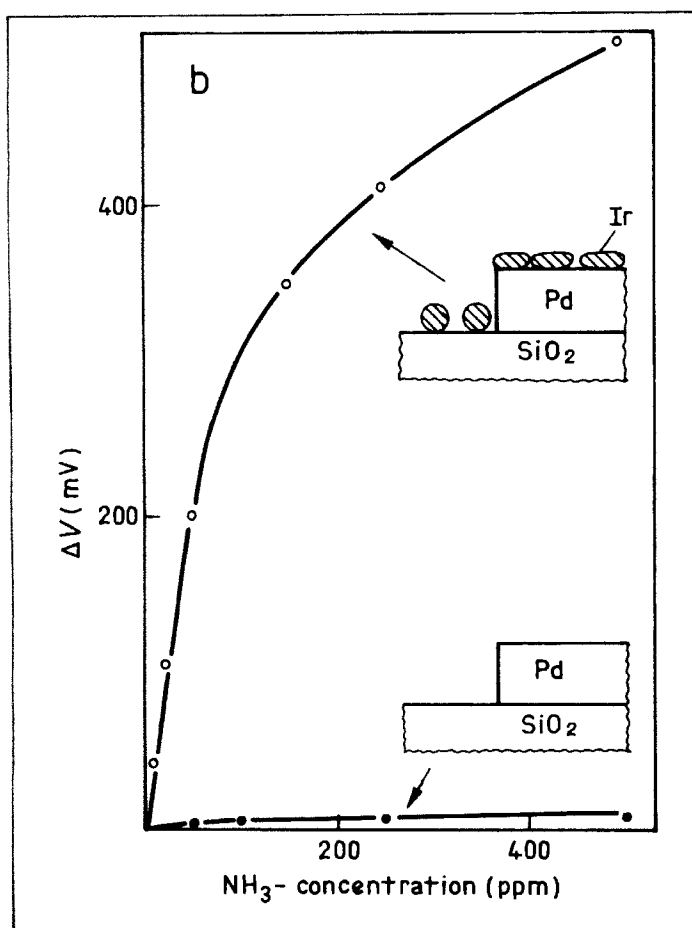
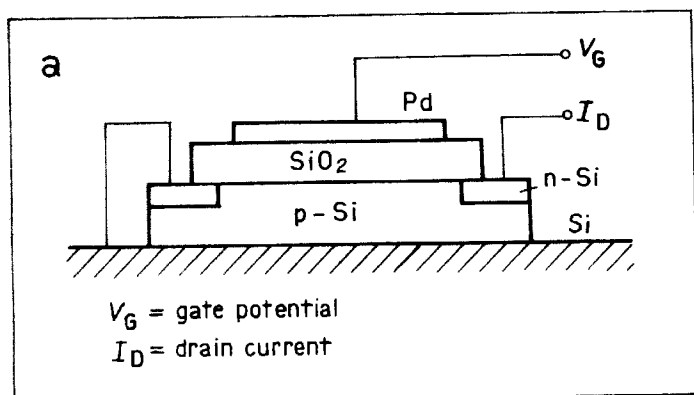


Fig. 12. Palladium MOSFET for measurement of ammonia. (a) Cross-sectional view of the Pd-MOSFET; (b) sensitivity of the MOSFET with and without iridium modification. (Redrawn from Lundström, 1978).

can be detected at sensitivities orders of magnitude higher than with conventional indication. Because there is no structure on the monolithic surface, the insulating layer is very stable.

Gas sensitive palladium metal oxide semiconductor structures (MOS) have been developed for the measurement of hydrogen and ammonia (Lundström, 1978). For indication of NH_3 the gate has been covered additionally with iridium (Fig. 12).

Microscale electrochemical devices having diode- or transistor-like characteristics may be constructed by the application of conducting polymers between microelectrodes (Fig. 13). In a microelectrochemical diode the polymer between the microelectrodes becomes conductive at a defined voltage; thus a 'breakthrough' occurs only above a certain value. A microelectrochemical transistor contains an additional electrode, the gate, controlling the redox state of the polymer (polypyrrole, polyaniline, or poly-3-methylthiophene). An alteration of the gate potential of only 0.5 V leads to a change of the resistance between the source and the drain by more than six orders of magnitude. In contrast to electronic transistors the resistance of microelectrochemical devices rises again when a certain, optimal gate potential is reached; therefore a potential increase switches the current first on and then off (Fig. 13b). Given the

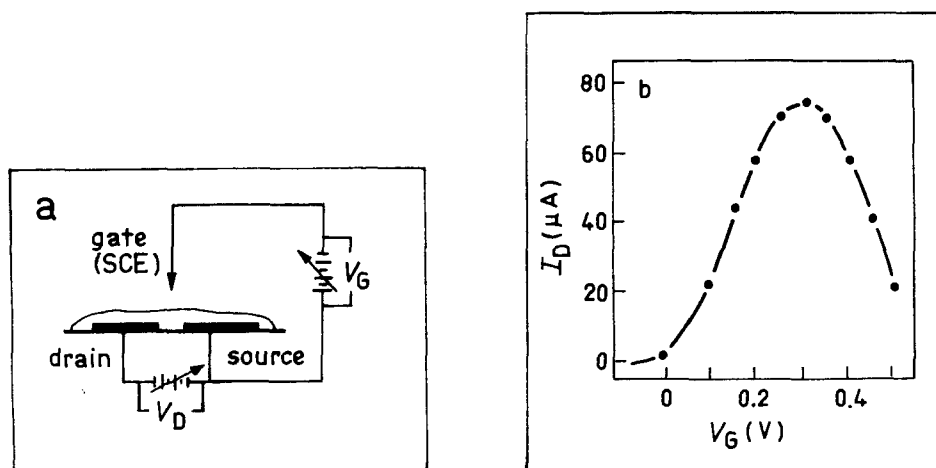


Fig. 13. Microelectrochemical device. (a) Schematic illustration of the microelectrochemical transistor based on polyaniline (thickness of the polyaniline layer 5 μm , electrode width 1–2 μm , distance 2–4 μm); (b) characteristic curve of the polyaniline transistor (I_D versus V_G at $V_D = 0.18$ V). (Redrawn from Wrighton, 1986).

possibility of transferring the redox potential change of enzymes directly to the polymer, these effects might be of the utmost importance for biosensor development, since they would greatly facilitate the construction of 'direct' potentiometric biosensors. Microsensors for oxygen and hydrogen have recently been developed by vapor deposition of polymers on the substrate (Wrighton, 1986).

2.2.4.2 Amperometric Electrodes

Amperometric sensors are based on heterogeneous electron transfer reactions, i.e., the oxidation and reduction of electroactive substances (Fig. 10). Oxygen and H_2O_2 , being the cosubstrate and the product of several enzyme reactions, as well as artificial redox mediators, such as ferricyanide, N-methylphenazinium ion (NMP^+), ferrocene, and benzoquinone may be determined amperometrically.

By an increase of the overvoltage, i.e. the deviation from the redox potential, the rate of the heterogeneous charge transfer process is enhanced so as to cause the rate of the whole process to become controlled by mass transfer. Under these conditions the diffusion current, I_d , is proportional to the concentration of the substance to be determined, S_0 :

$$I_d = n A F D_S S_0 / \delta$$

where δ = thickness of the diffusion layer (being constant at given convection), D_S = diffusion coefficient, A = electrode surface area, and n = number of exchanged electrons.

Detection limits as low as 10 nmol/l and a linear measuring range of 3–6 concentration decades are the main advantages of amperometric techniques.

The electrode potential is decisive for the selectivity of the sensor. Any electrode-active substance being electrochemically converted at lower potential contributes to the total current (Fig. 14). Thus, at an electrode potential of +600 mV for H_2O_2 indication ascorbic acid is oxidized as well. To eliminate these interferences, the working potential of the electrode is kept as low as possible. Therefore a reaction partner is chosen as an electrochemical indicator, which is converted at the lowest possible potential. As shown in the redox scheme of glucose oxidation (Fig. 15), in addition to oxygen several organic redox systems are able to reoxidize glucose oxidase (GOD), i.e., their redox potential is more positive than that of the prosthetic group. The anodic oxidation of the reduced mediators proceeds at an overvoltage of only 50–100 mV whereas the limiting current of hydrogen peroxide oxidation is reached

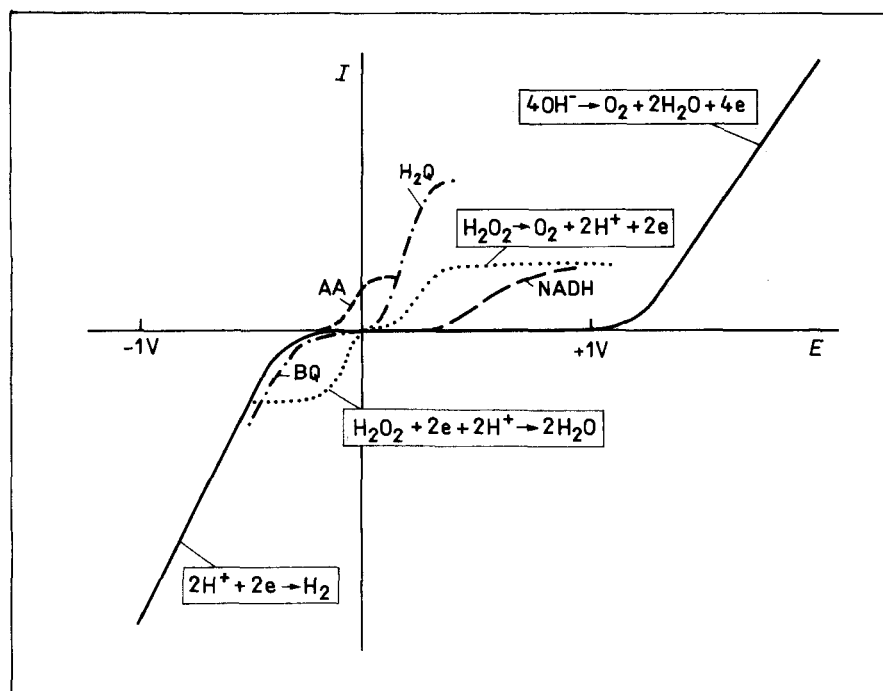


Fig. 14. Current potential curves of typical redox systems of amperometric enzyme electrodes. AA: ascorbic acid, H_2Q : hydroquinone.

only at +600 mV (see Fig. 14). Therefore, with these mediators an electrode potential may be applied that is lower by at least 400 mV; this eliminates any interference by ascorbic acid.

The *oxygen electrode* according to Clark is the most widely used amperometric electrode. It consists of a platinum cathode and an Ag/AgCl reference electrode, both being positioned in a KCl electrolyte solution. The Pt electrode is firmly covered by an O_2 -permeable polyethylene, polypropylene, or Teflon membrane of 10–50 μm thickness. The membrane separates the electrodes from the measuring solution (Fig. 16). The O_2 electrode is polarized against the reference electrode at -0.6 to -0.9 V, i.e. in the range of the diffusion current of cathodic oxygen reduction. Since interfering substances are excluded from the O_2 electrode, its selectivity is extraordinarily high. If the silver chloride is replaced by a base metal such as zinc or nickel, and an alkaline electrolyte is used, the potential difference necessary for cathodic oxygen reduction is created within the 'galvanic electrode' itself. Then no external potential source is required. To miniaturize the sensor, O_2 electrodes

based on vapor-deposited gold electrodes have been produced using microelectronic technology (Miyahara et al., 1983; Suzuki et al., 1988a).

Oxygen electrodes are being used for the direct determination of oxygen concentration in biomedicine and biotechnological processes. For the development of biosensors they can be combined with a large number of biocatalytic processes such as the respiration of microorganisms, plant photosynthesis, and oxidase-catalyzed reactions.

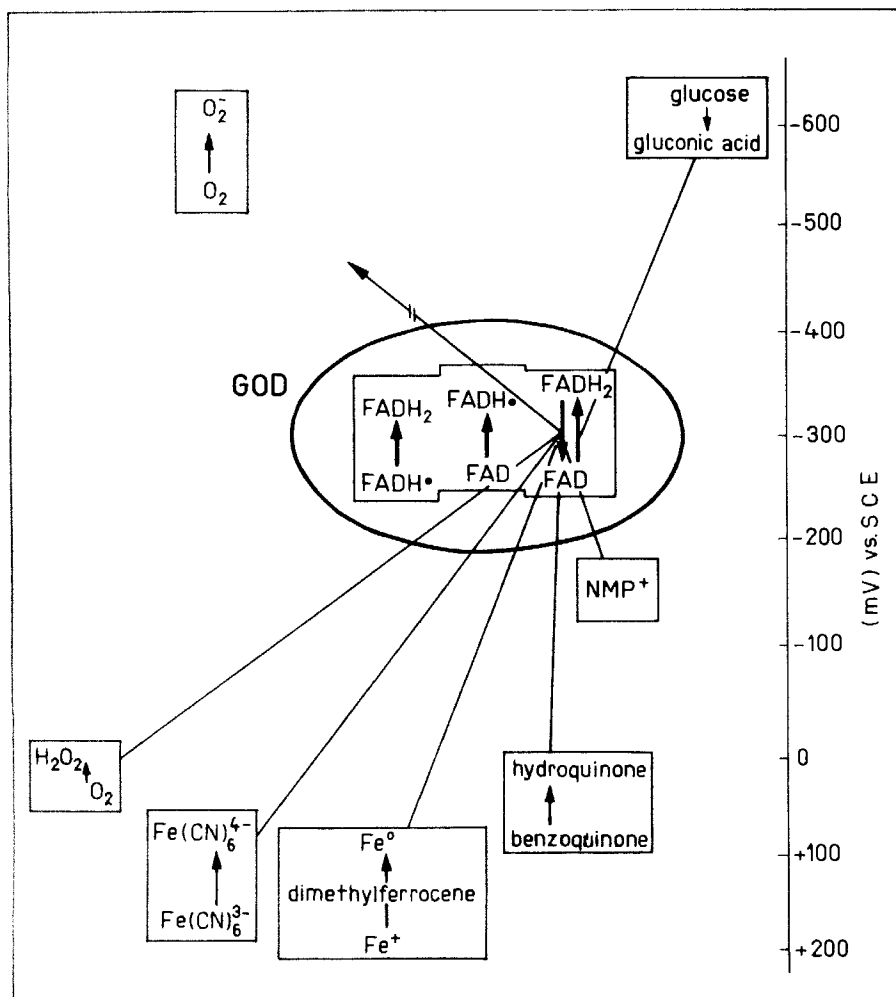


Fig. 15. Redox scheme of glucose oxidation in the presence of glucose oxidase. SCE: saturated calomel electrode, FAD: flavin adenine dinucleotide.

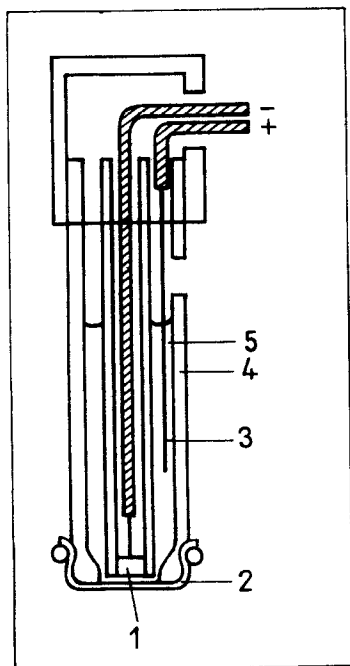


Fig. 16. Oxygen electrode according to Clark. 1: Platinum cathode, 2: polyethylene membrane, 3: silver anode, 4: electrode body, 5: potassium chloride solution.

Amperometric sensors for hydrogen, nitrous oxides, and carbon dioxide have been developed by modification of the Clark-type electrode (Hanus et al., 1980; Alberly and Barron, 1982).

When the Pt electrode of the previously described electrode configuration is polarized at +0.4 to +1.0 V, H_2O_2 can be measured with the sensor. Furthermore, the hydrophobic membrane has to be replaced by a dialysis membrane for this purpose. Since, at the potential of anodic hydrogen peroxide oxidation, various organic compounds, e.g. ascorbic acid, uric acid, glutathione, and NADH, are co-oxidized (see Fig. 14), the selectivity of the H_2O_2 -detecting electrode is relatively poor. Electrochemical interferences may in part be eliminated by covering the anode with a thin, anion-bearing membrane, e.g., one made from cellulose acetate or cellulose nitrate. An alternative may be the evaluation of the difference of the signals with and without the enzyme reaction. In spite of these problems amperometric enzyme electrodes based on oxidases in combination with H_2O_2 -indicating electrodes have become most common among biosensors.

As shown above for GOD, the natural electron acceptors of many enzymes, for example methanol dehydrogenase, alcohol dehydrogenase, cytochrome b_2 , and the oxidases of lactate, pyruvate, glycolate, sarcosine, and galactose, may be replaced by redox active dyes or other reversible redox systems (Fig. 17). This also enables the coupling of such enzymes with amperometric electrodes in oxygen-free solution (Albery et al., 1987; Frew and Hill, 1987; Taniguchi and Hawkrigde, 1988).

About 300 pyridine nucleotide dependent enzymes are currently known. Many of them are in widespread use for analytical purposes. Therefore the determination of the coenzyme NAD(P)H is of great importance. In contrast to the enzyme-catalyzed oxidation of NAD(P)H, its anodic oxidation proceeds in two separate one-electron steps with radical intermediates (Elving et al., 1982). It requires an overvoltage of about 1 V. Furthermore, electrode fouling by the reaction products makes the electrochemical process poorly reproducible. Owing to the high electrode potential, other oxidizable substances interfere signifi-

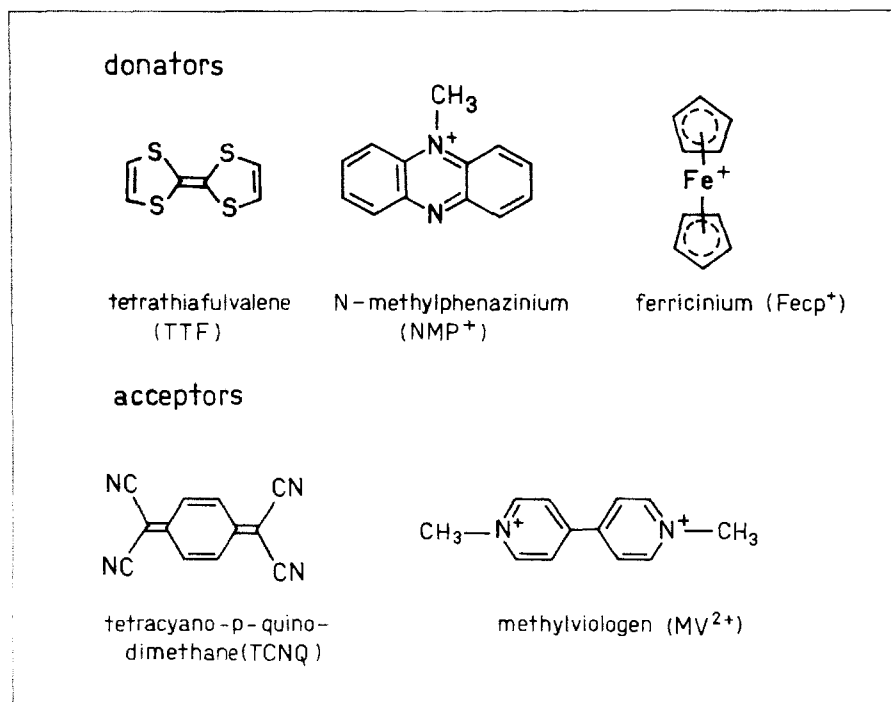


Fig. 17. Commonly used redox mediators.

cantly. To overcome these problems the following methods have been suggested:

- (i) anodic reoxidation of mediators reduced by NADH in the presence of diaphorase,
- (ii) measurement of O_2 consumption caused by autoxidation of mediators spontaneously reacting with NADH, e.g. NMP⁺ or FAD,
- (iii) aerobic oxidation in the presence of NADH oxidase,
- (iv) coupled oxidation with peroxidase,
- (v) NADH oxidation at modified electrodes, e.g., with TCNQ.

Direct anodic oxidation of highly diluted NADH-containing samples has also been successfully used (Eggers et al., 1982). Electrochemical indication of the oxidized coenzyme, $NAD(P)^+$, is only possible in deaerated solution and has not yet been utilized in biosensors.

A peculiarity of amperometric electrodes is the Faradaic conversion of a reaction partner. Thus, in enzyme electrodes based on O_2 measurement, the oxygen concentration at the electrode surface approximates zero. By substrate addition a part of the oxygen is consumed within the enzyme layer and the concentration profile is changed. In contrast, when H_2O_2 is indicated, a part of the oxygen that is enzymatically reduced to H_2O_2 is regenerated at the electrode. This means that the oxygen concentration is higher in the vicinity of the electrode.

The electrode reaction also offers the possibility of influencing the enzyme reaction (Fig. 18). In the O_2 -stabilized enzyme electrode dev-

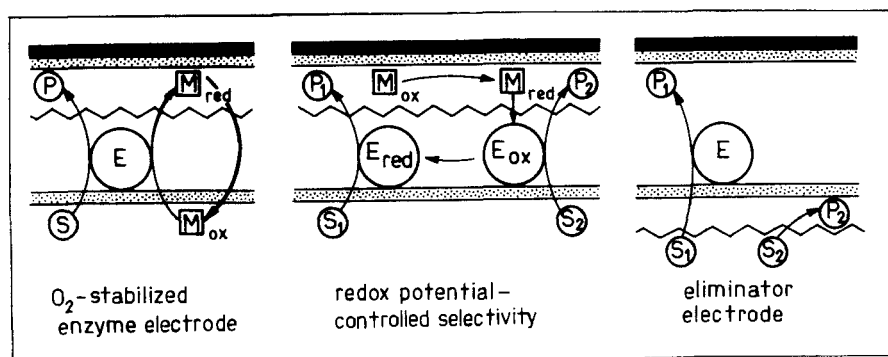


Fig. 18. Principles of electrochemical modulation of sensor characteristics by amperometric electrode reactions. E: enzyme, S: substrate; P: product; M: mediator.

eloped by Cleland and Enfors (1984) the influence of the oxygen content of the measuring solution is eliminated. For this purpose the same amount of oxygen as is consumed in the enzyme reaction is generated at a Pt net electrode arranged within the enzyme layer. This method is applicable to the determination of high substrate concentrations, at which the enzyme reaction is otherwise limited by oxygen.

The selectivity of galactose oxidase has been controlled by changing the redox potential in the enzyme layer with an auxiliary electrode (Fig. 18). The possibility of enhancing the specificity in this way was demonstrated by using substrate mixtures (Johnson et al., 1985). To eliminate interfering substances, an 'eliminator electrode' may be used for their quantitative conversion into non-disturbing products. This electrode type is made of a metal foil or net situated in front of the enzyme layer and indicator electrode. It has been used to suppress the ascorbic acid signal disturbing the anodic H_2O_2 indication (Scheller et al., 1985b).

A novel principle for increasing the selectivity is the use of an 'ion gate' membrane. Here, the ion permeability is controlled by electrochemical regulation of the redox state of the membrane material. For example, a polypyrrole film has to be in the reduced state to become permeable for anions. If the film is oxidized, no anion can permeate (Burgmayer and Murray, 1982).

Both the sensitivity and the specificity of a glucose microenzyme electrode have been improved by using voltage pulses (Ikariyama et al., 1988).

When mediators are used, e.g., for NADH oxidation or as artificial electron acceptors of enzymes, the following problems may occur:

- (i) The mediator concentration is in the millimolar range, causing high reagent costs.
- (ii) The interaction with interfering substances or microorganisms may falsify the measured value.
- (iii) Some mediators, e.g. ferrocene, are poorly soluble in water.

Therefore the concept of *chemically modified electrodes* has been developed, in which the mediator is integrated with the amperometric electrode (Fig. 19). The following methods for preparing mediator-chemically modified electrodes (MCME) have been described (Murray, 1984):

- (i) chemisorption of the mediator onto the electrode surface, e.g. by evaporation of a mediator-containing organic solvent (with ferrocene, TCNQ, TTF);

- (ii) entrapment of the redox system in a polymer film at the electrode, e.g. by electropolymerization of pyrrole;
- (iii) covalent fixation of the mediator to reactive groups of the electrode surface;
- (iv) inclusion of the mediator in carbon paste, which may be composed of carbon powder and paraffin oil;
- (v) formation of redox active groups by pretreatment of carbon electrodes;
- (vi) vapor deposition of noble metal monolayers on carbon;
- (vii) preparation of organic metal electrodes by using charge transfer complexes, e.g. of TCNQ^- or NMP^+ .

In a similar way to mediators, enzymes may be covalently bound to electrode surfaces, thus giving enzyme-chemically modified electrodes (ECME). When enzymes and mediators are coimmobilized, addition of auxiliary substances during the measuring process can be avoided: a reagentless measuring regime becomes feasible (Fig. 19).

An alternative to the application of mediators is the direct transfer of electrons between the prosthetic group of the enzyme and the amperometric electrode (Fig. 19). In this heterogenous reaction the electrode acts as an electron transferase.

Until the mid-70s it was generally agreed that redox active groups of proteins necessarily lose their catalytic function during electrochemical conversion, for the following reasons (Scheller and Strnad, 1982):

- (i) the strong adsorption to the electrode may cause structural changes leading to complete denaturation of the protein;
- (ii) the low diffusion coefficients of the macromolecules lead only to slow electrochemical conversion;

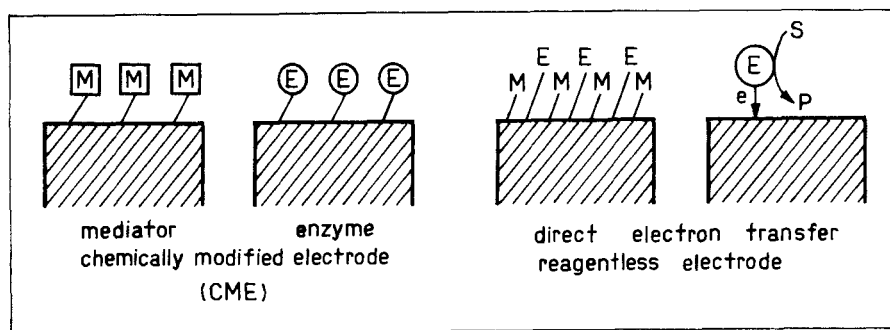


Fig. 19. Basic types of biochemically modified electrodes. Symbols as in Fig. 18.

- (iii) contaminations, such as dissociated prosthetic groups, may mask the electrochemical signal of the intact protein;
- (iv) the insignificance of the fraction of the prosthetic group in the whole macromolecule or its inaccessibility make a heterogenous electron transfer very inefficient.

Both the increasing availability of highly purified proteins and the development of chemically modified electrodes led to substantial progress in the field of protein electrochemistry. At present, heterogeneous electron transfer has been achieved with more than 30 different proteins; mainly electron transferases, but also substrate-converting oxidoreductases (Dryhurst et al., 1982). The following redox active groups of proteins have been electrochemically converted: disulfide bridges, sulfur iron clusters, flavin and heme groups, and several metal ions (Table 3). Whereas at metal electrodes the protein molecules are irreversibly bound in the first adsorption layer, for modified electrodes a rapid exchange with molecules from the solution has been postulated. Furthermore, the immobilized mediator is expected to promote an orientation of the protein at the electrode surface, which facilitates the electron transfer. This may be envisaged as an analogy to the mechanism of natural redox processes (Hill et al., 1982). In addition to this fundamental aspect, direct electron transfer attracts interest for practical application, namely for the development of reagentless sensors. Such sensors would be especially useful for on-line measurement in fermenters or as sensors to be implanted in living beings (Varfolomeev and Berezin, 1978).

The direct anodic oxidation of cytochrome *c* at a bipyridyl-modified electrode has already been incorporated in enzyme electrodes for lactate, carbon monoxide, and hydrogen peroxide. Here, cytochrome *c* is reduced by cytochrome *b₂*, CO oxidoreductase, or horseradish peroxidase and anodically reoxidized. Cytochrome *c* has also been applied to couple mitochondria and chloroplasts to redox electrodes (Albery et al. 1987). Although no practically applicable sensor has been constructed as yet, this principle offers a new avenue to the determination of inhibitors of photosynthesis or respiration (Cardosi and Turner, 1987).

An exciting method for accelerating the electron transfer between redox proteins and amperometric electrodes has been described by Heller and Degani (1987), who modified oxidoreductases with electron-tunneling relays. The same mediators as are used in chemically modified electrodes are directly bound to groups of the protein molecule. The

TABLE 3

Electrochemically Converted Proteins

Protein	Redox active group	Redox potential E_0 (mV vs SCE)	Half wave potential $E_{1/2}$ (mV vs SCE)	Electrode material
Cytochrome c	hemin	+16	+25	RuO ₂
Cytochrome c ₃	hemin	-516	-530	Hg
Cytochrome c ₇	hemin	-420	-435	Hg
Cytochrome b ₅	hemin	-240	-580	Hg
Cytochrome b ₂	hemin		-257	Pt
Cytochrome P-450	hemin	-560	-580	Hg
Azurine	Cu ²⁺	+27, +116	+86, +102	RuO ₂
Plastocyanine	Cu ²⁺	+116	+110	RuO ₂
Ferredoxin	FeS	-647	-630	RuO ₂
Flavodoxin	FMN	-550	-300, -600	Hg/PLL
Rubredoxin	FeS	-301	-310	RuO ₂
Insulin, ribonuclease, lysozyme, trypsin, chymotrypsin, egg albumin, serum albumin	disulphide		-600	Hg
Ribonuclease, concanavalin A, bovine serum albumin	tyr, trp		-950	carbon
Hydrogenase	4Fe/4S		-700	Hg/PLL
Laccase	Cu ²⁺	+165, +450	+410	graphite/DMP
Phosphorylase b	PLP		-820	Hg
Glucose oxidase	FAD	-300	-340	Hg
			-610	graphite
Xanthine oxidase	FeS	-534	-590	Hg
			-420, -520	graphite
Cholesterol oxidase	FAD		-360	Hg
L-amino acid oxidase	FAD		-510	graphite
Ferredoxin-NADP ⁺ oxidoreductase	FAD		-570	Hg
Horseradish peroxidase	hemin	-310	-710	Au/MV ²⁺
Methemoglobin	hemin	-70	-600	Hg, SnO ₂
Metmyoglobin	hemin	-236	-1050	Hg, Au/MV ²⁺

PLP — pyridoxalphosphate, DMP — 2,9-dimethyl phenanthroline, PLL — poly-L-lysine, MV — methylviologen, FMN — flavin mononucleotide, FAD — flavin adenine dinucleotide

small distance between the mediator molecules (at most 1 nm) provides for a very fast tunneling process. In order to fix the 'relays' near the prosthetic group the protein has to be unfolded. An enzyme electrode employing GOD modified in this way can be operated, like a mediator-modified electrode, without reagent addition.

2.2.4.3 Conductometric Measurement

Conductometric methods use non-Faradaic currents. An alternating current of low amplitude and a frequency in the range of 1 kHz is indicated. The measuring signal reflects the migration of all ions in the solution (see Fig. 10). It is therefore nonspecific and may only be used for samples of identical conductivity. Disturbances by deviating sample conductivity may be largely eliminated by combination of an enzyme-covered electrode pair with a pair of 'reference' electrodes covered by the carrier containing no enzyme. Such arrangements may be fabricated by small scale electronic production technology. An example has been the imprinting of Pt electrodes on ceramic substrates by the screen-printing technique (Watson et al., 1987/88). In conductometric membrane sensors the two electrodes are separated from the measuring solution by a gas-permeable membrane. The diffusion processes of the measured gases are the same as in gas sensitive ISEs. Likewise, the selectivity is improved by use of the membrane.

2.3 BIOCHEMICAL FUNDAMENTALS

The transducer determines mainly the *effectiveness* of signal processing and output of a biosensor. In contrast, the analytical *selectivity* of biosensors is determined by the specificity of the signal-producing interaction of the integrated biological component with the analyte. Moreover, the properties of the biomolecule, such as its specific activity or the material by which it is immobilized, influence the response time, the dynamic range, and the sensitivity of biosensors. In this respect account has to be taken of the fact that biological systems and processes are susceptible to deviations from their optimal environmental conditions; in particular, their thermal and chemical stability is limited. These peculiarities decisively determine the limits of applicability of biosensors.

Different biospecific interaction processes may be considered for the construction of biosensors. Enzymes, antibodies, lectins, hormones, microorganisms, organelles, or tissue sections can be used to act as molecular

recognition elements. Of all these, enzymes are the most important. However, the complementarity of *antibodies* and *antigens* as well as the recognition specificity of *membrane-bound receptor systems* are employed in biosensors to an increasing extent. These sensor types significantly differ from each other in the mechanism of signal formation.

2.3.1 Enzymes and Substrate Conversion

2.3.1.1 Structure and Catalytic Action

A prerequisite for the catalytic function of an enzyme is its native tertiary structure which is determined by the number and sequence of amino acids (primary structure) forming the molecule. Favoured by hydrogen bonds, parts of the polypeptide chain exist in an α -helical or a β -sheet structure (secondary structure). Most enzymes are globular proteins, the tertiary structure of which may be fixed by disulfide bonds between cysteine residues. A famous example is lysozyme (Fig. 20), consisting of 129 amino acids. A defined three-dimensional structure is

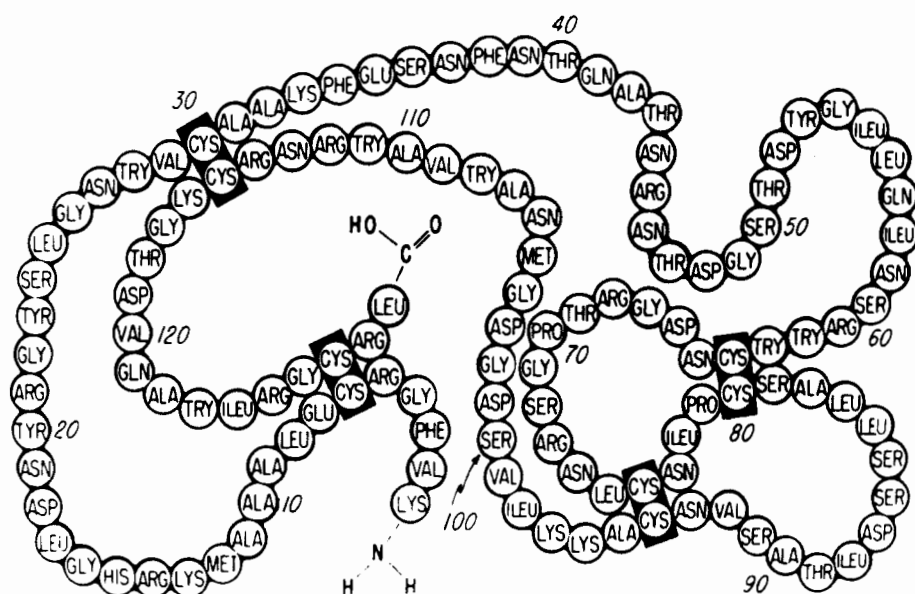


Fig. 20. Primary structure of hen-egg lysozyme. ALA: alanine, ARG: arginine, ASN: asparagine, ASP: aspartic acid, CYS: cysteine, GLN: glutamine, GLU: glutamic acid, GLY: glycine, HIS: histidine, ILE: isoleucine, LEU: leucine, LYS: lysine, MET: methionine, PHE: phenylalanine, PRO: proline, SER: serine, THR: threonine, TRP: tryptophan, TYR: tyrosine, VAL: valine. (Redrawn from Canfield and Lu, 1965).

further stabilized in aqueous solution by the formation of hydrophilic and hydrophobic regions from polar and apolar amino acid side chains and the inter- or intramolecular aggregation of these regions (quaternary structure, formation of micelles, etc.). In spite of these stabilizing interactions the ordered three-dimensional structure is only stable below 50°C and at medium pH. An exception are enzymes from microorganisms that have adapted to extreme environmental conditions, e.g., temperatures up to 90°C. Irreversible thermal inactivation at physiological pH is mainly due to hydrolytic cleavage of the amide side chain of asparagine, the peptide bond of aspartic acid, and the breaking of disulfide bridges (Ahern and Klibanov, 1986).

Within the mostly spherical, ellipsoidal, or kidney-shaped protein molecules a local cavity with a characteristic constitution and stereoconfiguration forms the catalytically active center (Fig. 21), where a chemically and spatially congruent substrate ('lock-and-key principle') is converted to a product. To a limited extent the protein structure is capable of adapting conformationally to the substrate.

Enzymes accelerate the equilibrium formation of chemical reactions by a factor of 10^8 – 10^{20} as compared with uncatalyzed reactions. Thus, urea is hydrolyzed at pH 8 and 20°C in the presence of urease about 10^{14} times faster than without catalysis, and the splitting of H_2O_2 is accel-

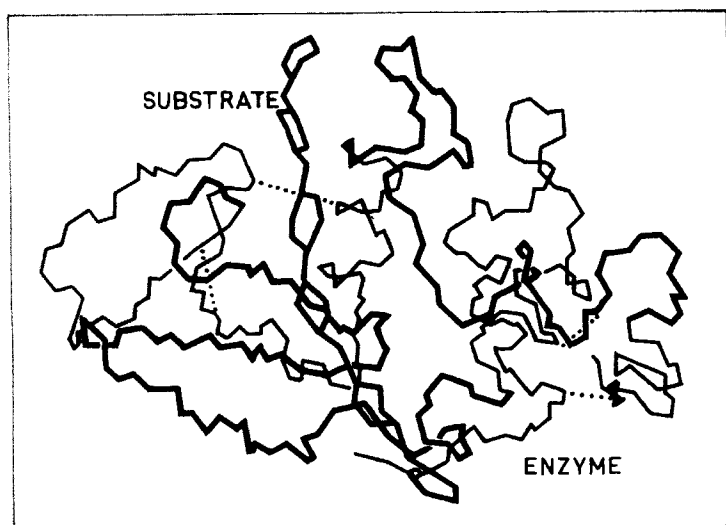


Fig. 21. Three-dimensional structure of lysozyme with the substrate bound to the active center (dotted lines are disulfide bonds).

erated by a factor of $3 \cdot 10^{11}$ in the presence of catalase. In other words, the cleavage of H_2O_2 normally requires an activation energy of 75.4 kJ/mol; catalase diminishes this energy to 23 kJ/mol (Hofman, 1984).

The activation energy can be determined by the Arrhenius equation:

$$k = Z e^{-E_a/RT}$$

where k stands for the rate constant, Z is a factor accounting for the frequency of collisions, and R is the gas constant. The activation energy, E_a , may be determined by measurement of the reaction rate at different temperatures and plotting $\ln k$ against $1/T$. For most biologically relevant reactions E_a amounts to 40–80 kJ/mol (Rapoport, 1977).

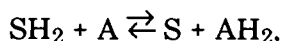
This drastic decrease of activation energy, being equivalent to a large increase of the number of reactive substrate molecules in the activated intermediate state, mostly results from molecular tensions induced in both components by the enzyme-substrate complex formation.

2.3.1.2 Classification of Enzymes

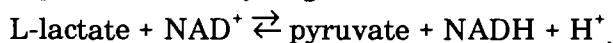
The roughly 3000 enzymes currently known are grouped into six main classes according to the type of the reaction catalyzed. At present only a limited number are used for analytical purposes.

Oxidoreductases catalyze oxidation and reduction reactions by transfer of hydrogen or electrons. The following are of analytical importance:

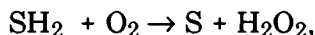
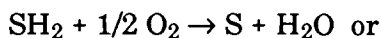
- (i) *dehydrogenases* catalyze hydrogen transfer from the substrate, S, to an acceptor, A (which is not molecular oxygen), or vice versa:



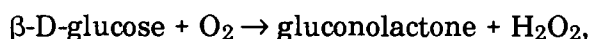
example: lactate dehydrogenase (EC 1.1.1.27)



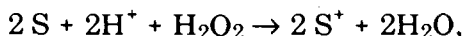
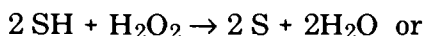
- (ii) *oxidases* catalyze hydrogen transfer from the substrate to molecular oxygen:



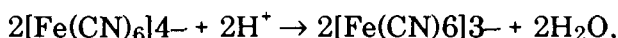
example: glucose oxidase (EC 1.1.3.4)



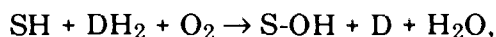
- (iii) *peroxidases* catalyze oxidation of a substrate by hydrogen peroxide:



example: horseradish peroxidase (EC 1.11.1.7)



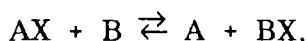
- (iv) *oxygenases* catalyze substrate oxidation by molecular oxygen with a hydrogen donor which may be the substrate itself:



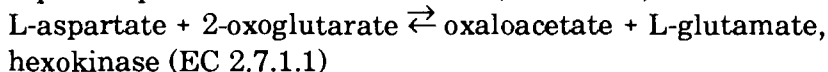
example: lactate-2-monooxygenase (EC 1.13.12.4)



Transferases transfer C-, N-, P-, or S-containing groups (alkyl, acyl, aldehyde, amino, phosphate, glycosyl) from one substrate to another. Transaminases, transketolases, transaldolases and transmethyldases belong to this class:



examples: aspartate aminotransferase (EC 2.6.1.1)

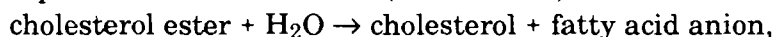


hexokinase (EC 2.7.1.1)

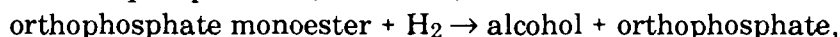


Hydrolases catalyze cleavages or the reverse fragment condensation. According to the type of bond cleaved, a distinction is made between peptidases, esterases, glycosidases, phosphatases, etc.

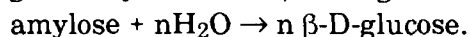
examples: cholesterol esterase (EC 3.1.1.13.)



alkaline phosphatase (EC 3.1.3.1)



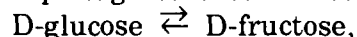
glucoamylase (exo-1,4- α -D-glucosidase) (EC 3.2.1.3)



Lyases nonhydrolytically remove groups from their substrates under formation of double bonds, or add groups to double bonds. Only few enzymes of this class are used in analysis.

Isomerases catalyze intramolecular rearrangements and are subdivided into racemases, epimerases, mutases, cis-trans-isomerases, etc.

examples: glucose isomerase (EC 5.3.1.5)



mutarotase (aldose-1-epimerase) (EC 5.1.3.3)

α -D-glucose \rightleftharpoons β -D-glucose.

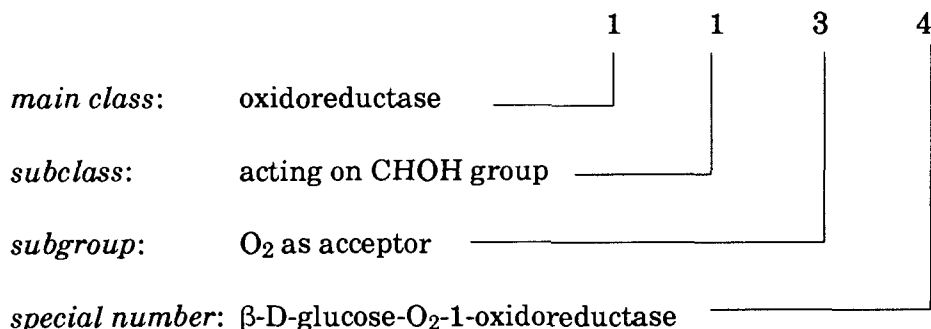
Ligases split C-C, C-O, C-N, C-S, and C-halogen bonds without hydrolysis or oxidation, mostly with the concomitant consumption of high-energy compounds like adenosine triphosphate (ATP) and other nucleoside triphosphates.

example: pyruvate carboxylase (EC 6.4.1.1)

pyruvate + HCO_3^- + ATP \rightleftharpoons oxaloacetate + ADP + P.

2.3.1.3 Enzyme Nomenclature

The systematic name of an enzyme consists of two parts, the first originating from the equation, the second from the type of reaction catalyzed. In addition, according to the recommendations of the International Union of Pure and Applied Chemistry and the International Union of Biochemistry (1973), each enzyme bears a number from the international EC (Enzyme Classification) system, which reflects the main class, the subclass, and the subgroup. The number is completed by a special enzyme number. Thus, for example the EC number 1.1.3.4 of the enzyme with the trivial name glucose oxidase results from the following:



For practical reasons, the trivial name is usually used.

2.3.1.4 Components Affecting the Action of Enzymes

In the development and application of biosensors based on enzymes several factors required for the catalytic process have to be taken into account, which are either directly involved in catalysis or influence the formation of the enzyme-substrate complex. They are designated *co-enzymes*, *prosthetic groups*, and *effectors*.

Coenzymes receive redox equivalents, protons, or chemical groups from the substrate during the enzymatic reaction. Since coenzymes readily dissociate from the enzyme they can act as group mediators between different enzyme molecules (e.g., coenzyme A). In case the factor is not reconverted to its original state by the *same* enzyme, it is called a cosubstrate. An example would be the cleavage of the energy-rich ATP to adenosine diphosphate (ADP) and phosphate during energy-consuming substrate conversion and the regeneration of the ATP by adenylate kinase. Many vitamin derivatives, such as coenzyme A, pyridoxalphosphate, thiamine pyrophosphate, and cobalamine (vitamin B₁₂), are coenzymes of enzymatic reactions (Table 4). Oxidative coenzymes with a defined redox potential serve as hydrogen or electron carriers during oxidoreduction reactions.

TABLE 4

Function of Some Important Coenzymes and Prosthetic Groups

Compound	Function
<i>(a) Oxidoreduction</i>	
Nicotinamide adenine dinucleotide	hydrogen transfer
Nicotinamide adenine dinucleotide phosphate	hydrogen transfer
Flavin mononucleotide	hydrogen transfer
Flavin adenine dinucleotide	hydrogen transfer
Heme (cytochromes)	electron transfer
Ferredoxins	electron transfer, hydrogen activation
<i>(b) Group transfer</i>	
Pyridoxalphosphate	transamination, decarboxylation, and others
Adenosine triphosphate	phosphate group donor
Tetrahydrofolic acid	C ₁ -group transfer
Biotin	carboxylation, decarboxylation
Coenzyme A	transacylation
Thiamine pyrophosphate (vitamin B ₁)	C ₂ -group transfer
Riboflavin	hydrogen transfer
5'-Deoxyadenosyl-cobalamine	transfer of methyl and other groups, isomerization

Prosthetic groups have the same function as coenzymes but are tightly bound to the enzyme. When they are split off, the protein is

mostly denatured. Flavin nucleotides and heme are the most important prosthetic groups (Table 4).

Effectors accelerate (activators) or block (inhibitors) the catalytic process. They are bound to the enzyme rather loosely and may therefore easily dissociate. Many of them are metal ions, e.g. Mg^{++} , Ca^{++} , Zn^{++} , K^+ , and Na^+ , which either form stoichiometric complexes with the substrate, stabilize an optimal protein conformation, or effect the association of subunits. These inorganic complements of enzyme reactions are frequently subsumed together with coenzymes as cofactors.

2.3.1.5 Kinetics of Enzyme-Catalyzed Reactions

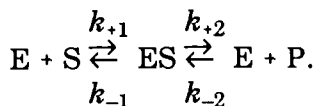
The time course of an enzymatic reaction permits one to deduce the substrate affinity, the catalytic mechanism in the active center, and the efficiency of the enzyme (maximum rate, turnover number).

The rate of an enzyme-catalyzed single reactant reaction depends on the concentration of substrate and product, respectively:

$$v = -dS/dt = dP/dt.$$

The initial rate (v_0) is determined by extrapolating the slope of the time course of the substrate or product concentration to time zero (Fig. 22). The dependence of v_0 on the substrate concentration, S (at constant enzyme concentration), is shown in Fig. 23. It reflects the typical substrate saturation. At first, v_0 increases proportionally to the amount of substrate. Upon further enhancement of substrate concentration v_0 levels off. The curve asymptotically approaches a maximum value, v_{\max} . When this plateau is reached, a change of S does not lead to a measurable change of v_0 : the enzyme is saturated by substrate and has thus reached the limit of its efficiency.

These kinetics result from the fast and reversible formation of an enzyme-substrate complex, ES , which dissociates in a second, slower reaction under liberation of the product, P :



Because the second reaction is rate-limiting, at very high substrate concentration almost all enzyme is present as enzyme-substrate complex. Under these conditions a steady state is reached in which the enzyme is steadily saturated by substrate and the initial rate is at a

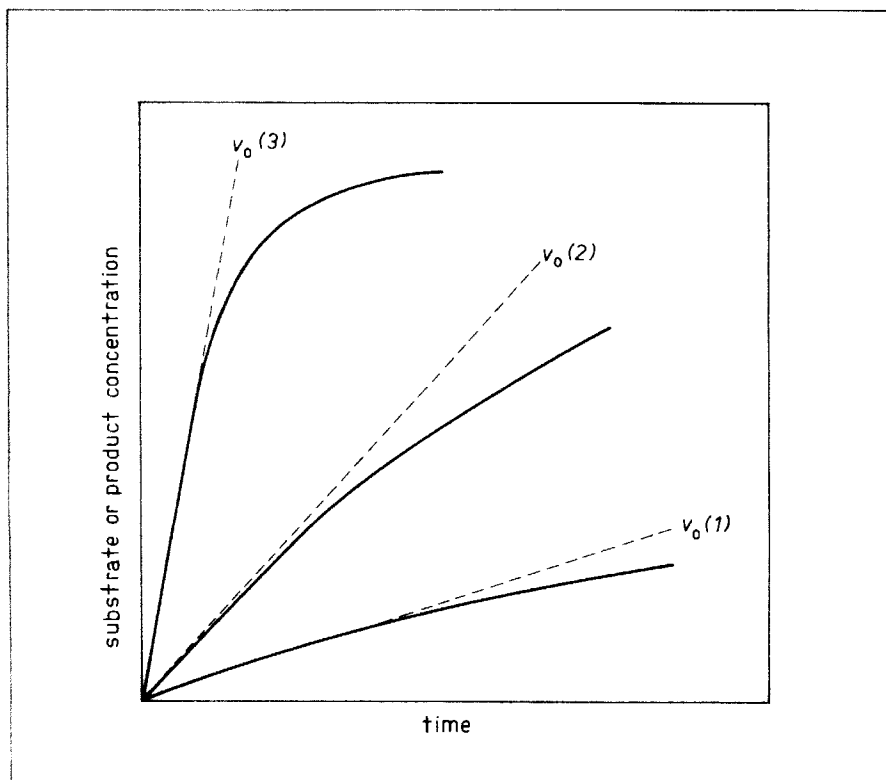


Fig. 22. Determination of the initial rate, v_0 , of an enzyme reaction. $v_0(1)$, $v_0(2)$, and $v_0(3)$ are initial rates under different conditions, e.g., different enzyme concentrations.

maximum (v_{\max}). This relation between substrate concentration and reaction rate may be described by the Michaelis–Menten equation:

$$v_0 = \frac{v_{\max} S}{K_M + S}$$

where K_M is the Michaelis constant of the enzyme for the given substrate. K_M may also be described by

$$K_M = \frac{k_{-1} + k_{+2}}{k_{+1}}$$

The relevance of K_M becomes evident at $S = K_M$. Then $v_0 = 1/2 v_{\max}$, i.e., K_M is the substrate concentration at which the reaction rate is half maximum (Fig. 23).

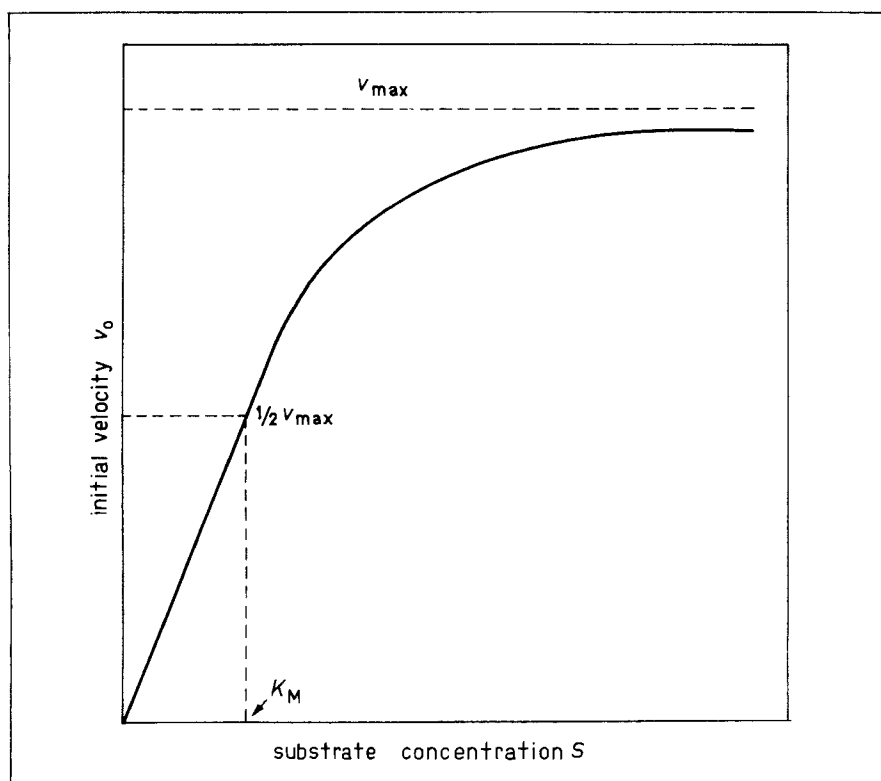


Fig. 23. Dependence of the initial rate of an enzyme-catalyzed reaction, v_0 , on the concentration of substrate, and derivation of the Michaelis constant.

The K_M value characterizes the affinity between the substrate and the enzyme. At known K_M and v_{\max} , v_0 can be calculated for each value of substrate concentration.

A low K_M value reflects high affinity. At substrate concentrations $S \ll K_M$ the reaction rate is directly proportional to the substrate concentration (first order reaction); at high substrate concentration ($S \gg K_M$) the reaction is zero order and is no longer dependent on the substrate concentration but only on the enzyme activity. To calculate K_M and v_{\max} as well as inhibitor constants it is advantageous to transform the Michaelis–Menten relation so as to obtain linear relationships between S and v_0 that can be evaluated graphically. An example is the Lineweaver–Burk equation, containing the reciprocal values of v_0 and S :

$$\frac{1}{v_0} = \frac{1}{v_{\max}} \left(1 + \frac{K_M}{S} \right)$$

The relevant plot gives K_M and v_{\max} at the intercepts of the curve with the abscissa and the ordinate, respectively (Fig. 24).

Most enzyme reactions involve more than one substrate. If the reaction requires the binding of every substrate via a ternary complex before any product can be released, it has a sequential mechanism. The initial velocity, v_0 , of such a two-substrate reaction can be expressed by the following equation:

$$v_0 = v_{\max} \cdot A \cdot B / K_i^A \cdot K_M^B \cdot A + K_M^A \cdot B + A \cdot B$$

v_{\max} represents the maximum velocity when the concentration of both substrates, A and B , is saturating. K_M^A is defined as the limiting Michaelis constant of A when B is saturating, K_i^B is the limiting value of K_M when B becomes zero.

For a ping-pong mechanism, in which some products are released before all substrates have been bound, the following equation is valid:

$$v_0 = v_{\max} \cdot A \cdot B / K_M^B \cdot A + K_M^A \cdot B + A \cdot B$$

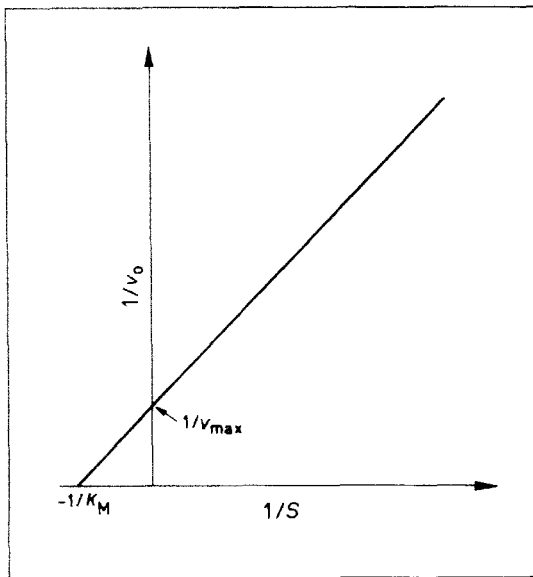


Fig. 24. Graphic estimation of the Michaelis constant, K_M , and the maximum rate, v_{\max} , according to Lineweaver and Burk.

When the (one-substrate) Michaelis–Menten equation is applied at saturation of B the following apparent parameters are obtained for the sequential mechanism:

$$K_M^A(\text{app}) = \frac{K_i^A \cdot K_M^B + K_M^A \cdot B}{K_M^B + B}$$

$$v_{\max}(\text{app}) = \frac{v_{\max} B}{K_M^B + B}$$

and for the ping-pong mechanism:

$$K_M^A(\text{app}) = \frac{K_M^A \cdot B}{K_M^B + B}$$

$$v_{\max}(\text{app}) = \frac{v_{\max} B}{K_M^B + B}$$

2.3.1.6 Enzyme Activity and Enzyme Concentration

In addition to K_M and v_{\max} , the *turnover number* (molar activity) and the *specific activity* are important parameters for the characterization of enzyme reactions. Both are determined under substrate saturation. With highly purified enzymes the turnover number reflects the number of substrate molecules converted in unit time by a single enzyme molecule (or a single active center). Catalase, one of the most potent enzymes, has a turnover number of $2 \cdot 10^5/\text{s}$.

The specific activity of enzymes is given in units. One international unit (IU) is the amount of enzyme consuming or forming 1 μmol substrate or 1 μmol product per minute under standard conditions. The base unit is 1 katal, corresponding to the amount of enzyme converting 1 mol substrate per second:

$$1 \text{ kat} = 6 \cdot 10^7 \text{ IU},$$

$$1 \text{ IU} = 16.67 \text{ nkat}.$$

Usually U is used instead of IU.

For the quantitative determination of enzyme activity, initial rates are measured at different enzyme concentrations and near substrate saturation, in a suitable temperature range (25–37°C) and at optimal

pH. In a certain range the enzyme activity is proportional to the enzyme concentration. The enzyme activity of a sample can be estimated from the linear part of the plot.

2.3.1.7 pH and Temperature Dependence

Each enzyme has a characteristic pH optimum at which its activity is at a maximum. In the range of this optimum essential proton-donating or proton-accepting groups in the active center of the enzyme are in the ionized state required for the enzyme to function. Outside this range, substrate binding is no longer possible, and at extreme pH values the enzyme may be irreversibly denatured. The pH optimum depends on the composition of the medium, the temperature, and the enzyme's stability in acid and alkaline environments. The pH stability does not necessarily coincide with the pH optimum of the reaction rate.

As with all chemical reaction rates, those of enzyme reactions increase with increasing temperature (by a factor of 1.4–2.0 per 10 K), a limit being set by the thermal stability of the protein. The optimum temperature may be in a wide range, roughly between 30 and 80°C.

2.3.1.8 Inhibition of Enzyme Reactions

The function of enzyme-based biosensors may be severely restricted by inhibitors. The inhibition is either reversible or results in an irreversible inactivation of the enzyme.

Inhibitors structurally related to the substrate may be bound to the enzyme active center and compete with the substrate (competitive inhibition). If the inhibitor is not only bound to the enzyme but also to the enzyme-substrate complex, the active center is usually deformed and its function is thus impaired; in this case the substrate and the inhibitor do not compete with each other (noncompetitive inhibition). Competitive and noncompetitive inhibition effect the enzyme kinetics differently. A competitive inhibitor does not change v_{\max} but increases K_M (Fig. 25a); in contrast, noncompetitive inhibition results in an unchanged K_M and an increased v_{\max} (Fig. 25b). Some enzymes, e.g. invertase, are inhibited by high product concentration (product inhibition).

2.3.1.9 Isozymes and Allosteric Enzymes

Many enzymes exist in more than one molecular form. These isozymes catalyze the same reaction but exhibit different kinetic properties (K_M and v_{\max} values) and amino acid sequences. This means that their structural differences are genetically determined. Thus, all isozymes of

lactate dehydrogenase (LDH) consist of four subunits but there are two different polypeptide chains, namely the muscle (M) type and the heart (H) type, having different amino acid sequences. Therefore several LDH isozymes may be formed (M_4 , M_3H_1 , M_2H_2 , M_1H_3 and H_4). High LDH- H_4 activity in serum, for example, indicates heart tissue damage.

Allosteric enzymes are oligomeric proteins exhibiting a sigmoidal dependence of the reaction rate on substrate concentration instead of a 'normal', hyperbolic (classical Michaelis–Menten) one. At first the rate increases only slightly with increasing substrate concentration; there is then a rapid increase until near the maximum rate. This behaviour results from the presence of a regulatory allosteric center located on the same or another subunit as the catalytic center. This center may interact

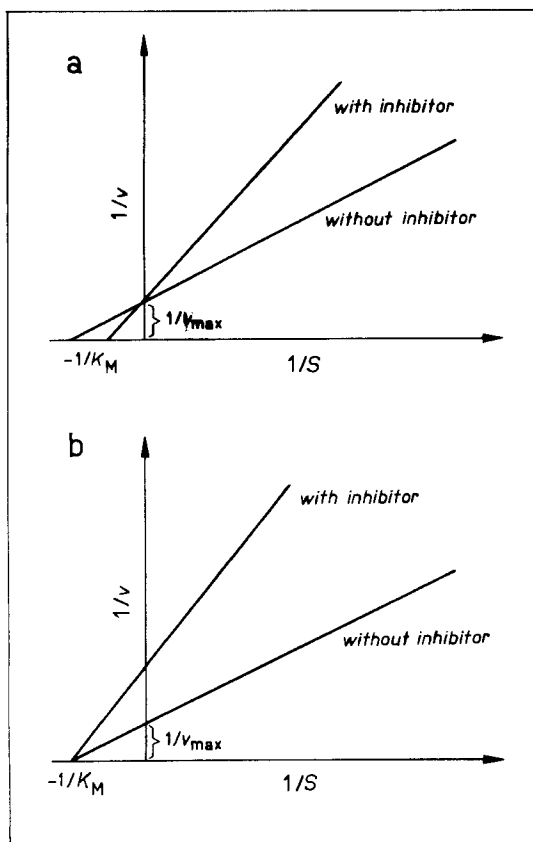
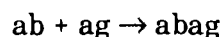


Fig. 25. Kinetics of enzyme-catalyzed reactions with (a) competitive, and (b) non-competitive inhibition.

with an effector, such as a reaction product (feedback mechanism). The resulting conformational changes may inhibit or activate the function of the catalytic center. An allosteric enzyme can be controlled by one or more specific effectors. Besides reversible interactions, covalent binding of the effector is also possible. These properties of allosteric enzymes make their application in biosensors rather difficult.

2.3.2 Antibody–Antigen Interaction

Antibodies (ab) are high-molecular weight soluble proteins (immunoglobulins) produced by organisms in response to foreign substances (antigens, ag), with whom they form immunochemical complexes:



Immunoglobulins are subdivided into classes having distinct molecular weights and properties (IgG, IgM, IgA, IgD, and IgE). Each immunoglobulin consists of two 'light' and two 'heavy' peptide chains connected by disulfide bonds. The antibody molecule has the shape of a Y on the upper parts of which two antigen binding sites are located. As with the binding between enzyme and substrate, that of antibody and antigen proceeds according to the lock-and-key principle. It is determined by relatively weak noncovalent forces such as hydrogen bonds, hydrophobic or Van der Waals forces and ionic interactions. An affinity constant, K , is used to describe the strength of the interaction. K is equal to the reciprocal concentration of free antigen necessary to occupy half of the antigen binding sites of the antibody. It is usually between $5 \cdot 10^4$ and 10^{12} l/mol.

Although the antibody–antigen binding is highly specific and therefore attractive for the construction of biosensors, up to now the direct measurement of this interaction has barely been possible. This is why most biosensors with antibody–antigen recognition are based on the principle of enzyme immunoassay (Mattiasson, 1977; Gebauer and Rechnitz, 1982): a known amount of enzyme-labeled antigen is added to a sample with unknown antigen concentration. When this mixture reacts with antibody, enzyme-labeled and non-labeled antigens compete for the binding sites of the antibody. The more antigen there is in the sample, the lower is the fraction of enzyme-labeled antigen in the antigen–antibody complex. After removal of unbound antigen the amount of bound enzyme-labeled antigen is determined via the enzyme-catalyzed reaction. Widely used indicator enzymes are horseradish

peroxidase and alkaline phosphatase. Urease and deaminases have also been used in immunosensors (Meyerhoff and Rechnitz, 1979; Gebauer and Rechnitz, 1982).

2.3.3 Function of Receptors

Biological receptors are protein molecules with a specific affinity for hormones, antibodies, enzymes, and other biologically active compounds; most of them are bound to the cell membrane. A receptor-ligand interaction is transmitted to other molecules inside the cell, where consecutive reactions are triggered. The currently best-known receptors are those for hormones. Many hormones released into the blood, e.g. insulin, glucagon, or adrenaline, do not penetrate the cell membrane but react with specific receptors at the cell surface. These are present in enormously high amounts, e.g., a single fat cell of about 50 μm diameter carries 160 000 insulin receptors, which corresponds to about 20 receptors per μm^2 . The receptor molecules mostly penetrate the cell membrane and many of them are coupled inside to an enzyme system. A conformational change of the receptor molecule by hormone binding may be directly mediated to the enzyme and result in its activation. Thus, for example the adrenaline receptor at the surface of the liver cell reacts with adrenaline formed in the adrenocortex and released into the blood under stress. The resulting conformational change of the receptor molecule activates associated adenylate cyclase reaching into the internal space of the cell and converting ATP to cyclic adenosine monophosphate (cAMP). cAMP initiates the phosphate transfer from ATP to other enzymes by protein kinase. In this way a number of other enzyme reactions are started, leading to a cascade of activated enzymes. Finally, a single adrenaline molecule will have stimulated several thousands of enzyme molecules, which will themselves liberate about three million glucose molecules from glycogen within a few seconds. An extremely weak chemical signal is thus immediately enzymatically amplified a millionfold and the sugar reserve of the body is mobilized.

In addition to hormone receptors, taste and olfactory receptors are typical examples of this biospecific recognition process. Presumably there are about 20 to 30 primary smells. After being bound to the appropriate receptor, their molecules cause conformational changes in the receptor molecule leading to a depolarization of a part of the nerve cell membranes and initiating an action potential.

Another receptor type is that of light receptors. The retina of the

human eye contains about 10^8 tightly packed receptor cells. Here, biochemical reactions, namely of the rhodopsin molecule, are not initiated by the binding of chemical substances but by light quanta. The reactions are enzymatically amplified and transformed into electrical impulses via membrane potential changes. Because of its light-absorbing chemical group the protein bacteriorhodopsin from salt-tolerant halobacteria has been studied in detail as a photoreceptor model. A single photon is sufficient to give rise to a conformational change of the protein and to transport two protons outside the cell. This 'proton pump' forms a proton and voltage gradient across the cell membrane driving the production of energy-rich ATP.

Compared to the investigation of enzymes, that of the structure and function of membrane-bound receptors and their biotechnological application is only at the very beginning. An analogous classification, e.g., according to recognition mechanism or specificity has not yet been attempted. However, any progress in this field will provide impetus to the development of new biosensors based on receptors.

2.4 IMMOBILIZATION OF THE RECEPTOR COMPONENT IN BIOSENSORS

For the repeated use of enzymes, cells, antibodies, and other biologically active agents in analytical devices, numerous techniques for fixing them to carrier materials have been developed. Immobilization, particularly of enzymes, brings about a number of further advantages for their application in analytical chemistry:

1. in many cases the enzyme is stabilized;
2. the enzyme-carrier complex may be easily separated from the sample, i.e., the latter is not contaminated by the enzyme preparation;
3. the stable and largely constant enzyme activity renders the enzyme an integral part of the analytical instrument.

2.4.1 *Methods of Immobilization*

The techniques for immobilizing biologically active agents comprise physical and chemical methods as well as combinations of both. The main physical methods are adsorption to water-insoluble carriers and entrapment in water-insoluble polymeric gels. Chemical immobilization

is effected by covalent coupling to derivatized carriers or by intermolecular crosslinking of the biomolecules (Fig. 26). Typical examples will be described in connection with the glucose sensor (Section 3.1.1).

Any of these methods has numerous variants; the suitability of a method for a particular task is at present still being empirically elucidated. However, some generally valid aspects will be outlined below.

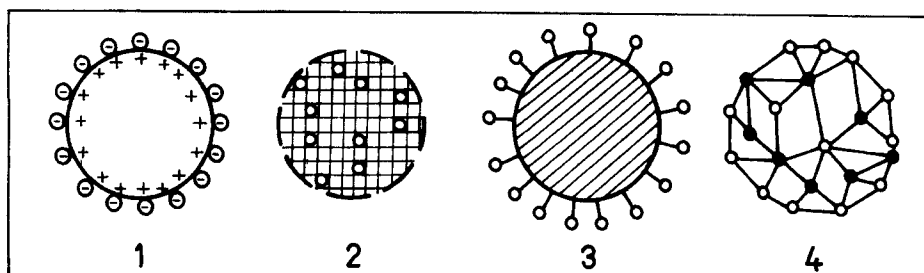


Fig. 26. Schematic representation of immobilization methods used for biosensor construction. ○: enzyme molecule, ●: crosslinker molecule, 1: adsorption, 2: gel entrapment, 3: covalent binding to external surface, 4: crosslinking.

2.4.1.1 Adsorption

The adsorption of biomolecules onto carriers that are insoluble in water is the simplest method of immobilization. An aqueous solution of the biomolecules is contacted with the active carrier material for a defined period of time. Thereafter the molecules that are not adsorbed are removed by washing. Anionic and cationic ion exchange resins, active charcoal, silica gel, clay, aluminum oxide, porous glass, and ceramics are being currently used as active material. The carrier should exhibit high affinity and capacity for the biomolecule and the latter must remain active in the adsorbed state. The carrier should adsorb neither reaction products nor inhibitors of the biocatalyst.

Since the adsorption of a protein to a surface is basically a reversible process, changes of pH, ionic strength, substrate concentration, temperature, etc. may detach the biomolecule from the carrier (Carr and Bowers, 1980). In addition to the simplicity of the procedure, the advantage of adsorptive immobilization is that it does not need nonphysiological coupling conditions or chemicals potentially impairing enzyme or cell functions. An activity loss is therefore seldom observed.

2.4.1.2 Gel Entrapment

Entrapment in polymeric gels prevents the biomolecules from diffusing from the reaction mixture. On the other hand, small substrate and effector molecules can easily permeate. Gel entrapment is as mild a procedure as adsorption, i.e., the biomolecules are not covalently bound to the matrix, membrane, or to each other. The method is therefore widely employed. The most important matrices used are alginate, carrageenan, collagen, cellulose triacetate, polyacrylamide, gelatin, agar, silicon rubber, poly(vinyl alcohol), and prepolymers crosslinked by addition of water (e.g. polyurethanes) or light.

2.4.1.3 Covalent Coupling

To covalently couple biomolecules, such as enzymes or antibodies, to carriers the dissolved protein is either reacted with an activated (e.g. functionalized) water-insoluble carrier or copolymerized with a reactive monomer. The reaction should involve only groups that are not essential for the biological activity of the biomolecule. Chemically reactive sites of a protein may be amino groups, carboxyl groups, phenol residues of tyrosine, sulfhydryl groups or the imidazole group of histidine. The immobilization is conducted in three steps: activation of the carrier, coupling of the biomolecule, and removal of adsorbed biomolecules. A disadvantage of covalent coupling is the frequently occurring loss of activity. Carriers are water-insoluble polysaccharides (cellulose, dextran, agarose derivatives) and high-molecular weight proteins (collagen, gelatin, albumin) as well as synthetic polymers (poly(vinyl chloride), ion exchange resins) and inorganic materials (porous glass).

2.4.1.4 Crosslinking

Biopolymers may be intermolecularly crosslinked by bi- or multifunctional reagents. The protein molecules may be crosslinked with each other or with another, functionally inert protein (e.g. albumin or gelatin). The biomacromolecules can also be adsorbed to a water-insoluble carrier or entrapped in a gel and then crosslinked. Among others, glutaraldehyde, bisisocyanate derivatives, and bisdiazobenzidine are being used as bifunctional reagents.

The advantages of crosslinking are the simple procedure and the strong chemical binding of the biomolecules. Furthermore, the choice of the degree of crosslinking permits the physical properties and the particle size to be influenced. The main drawback is the possibility of activity losses due to chemical alterations of the catalytically essential sites of the protein.

2.4.2 Immobilization Effects in Biosensors

Both for economical reasons and in order to achieve a high sensitivity and functional stability, immobilization methods having a high activity yield are desirable for biosensors. In the preparation of the biocomponent of a sensor various factors affecting this yield have to be taken into account.

The *measurable activity* reflects the biocatalytic efficiency of an immobilized enzyme. In homogeneous solution the initial rate of substrate conversion rises linearly with enzyme concentration. The reaction rate is influenced by substrate diffusion only at extremely large degrees of conversion. With *immobilized enzymes* the measured reaction rate depends not only on the substrate concentration and the kinetic constants K_M and v_{\max} but also on so-called immobilization effects. These effects are due to the following alterations of the enzyme by the immobilization process (Kobayashi and Laidler, 1974).

1. Conformational changes of the enzyme caused by immobilization usually decrease the affinity to the substrate (increase of K_M). Furthermore, a partial inactivation of all, or the complete inactivation of a part of the enzyme molecules may occur (decrease of v_{\max}). These two cases of a conformation-induced drop of v_{\max} may be distinguished by measuring the activity of the resolubilized enzyme or by titration of the active center with an irreversible inhibitor.
2. Ionic, hydrophobic, or other interactions between the enzyme and the matrix (microenvironmental effects) may also result in changed K_M and v_{\max} values. These essentially reversible effects are caused by variations in the dissociation equilibria of charged groups of the active center.
3. A non-uniform distribution of substrate and/or product between the enzyme matrix and the surrounding solution effects the measured (apparent) kinetic constants.
4. In biosensors the biocatalyst and the signal transducer are spatially combined, i.e., the enzyme reaction proceeds in a layer separated from the measuring solution. The substrates reach the membrane system of the biosensor by convective diffusion from the solution. The rate of this external transport process depends essentially on the degree of mixing. In the multilayer system in front of the sensor the substrates and products are

transferred by diffusion. Slow mass transfer to and within the enzyme matrix leads to different concentrations of the reaction partners in the measuring solution and in the matrix. Diffusion and the enzyme reaction do not proceed independently of one another; they are coupled in a complex manner.

The theory of the coupling of enzyme-catalyzed reactions with transport processes has been investigated for the following limiting cases (Carr and Bowers, 1980):

1. external diffusion limitation by mass transfer through layers in front of the enzyme membrane, e.g., the diffusion layer at the interface solution/biosensor, or a semipermeable membrane;
2. internal limitation by diffusion within the enzyme layer or by the enzyme reaction.

Usually in the operation of biosensors the flow conditions are adjusted to provide a mass transfer rate from the solution to the membrane system which is fast as compared with the internal mass transfer (exception: implanted sensors). On the other hand, variations of the diffusion resistance of the semipermeable membrane are being used to optimize the sensor performance. A semipermeable membrane with a molecular cutoff of 10 000 and a thickness of 10 μm only slightly influences the response time and sensitivity. In contrast, thicker membranes, e.g. of polyurethane or charged material, significantly enhance the measuring time, but may also lead to an extension of the linear measuring range.

For biosensors based on transducers that do not consume cosubstrate or product (e.g. potentiometric electrodes or optoelectronic detectors), Blaedel et al. (1972) derived the following relation between the product concentration at the transducer surface, P^d , and the substrate concentration in the measuring solution, S^0 :

$$P^d = S^0 \frac{D_S}{D_P} (1 - \text{sech} \sqrt{f_E}) \text{ for } S^0 \ll K_M,$$

$$\text{with } f_E = \frac{v_{\max} d^2}{K_M D_S},$$

$$v_{\max} = k_{+2} E.$$

Provided external diffusion is not limiting, P^d depends linearly on S^0

and the ratio of the substrate and product diffusion coefficients (D_S and D_P), and nonlinearly on a root expression, the so-called Thiele-modulus (the square of which is termed the enzyme loading factor, f_E).

The latter parameter reflects the ratio of the rate of the enzymatic reaction, v_{\max}/K_M , to that of diffusion, D_S/d^2 , d being the thickness of the enzyme layer. It indicates whether the process in an enzyme layer is determined by enzyme kinetics or by substrate diffusion. At $f_E < 25$ the process is *kinetically controlled*. In this case the substrate concentration does not become zero in any part of the enzyme layer, i.e., the enzyme sensor signal is mainly a function of the 'active' enzyme concentration. Therefore, effectors (activators, inhibiting factors, including H^+ and OH^-) and the enzyme loading, i.e., the amount of enzyme in front of the transducer, as well as the time-dependent enzyme inactivation, all directly effect the measuring signal.

At $f_E > 25$ *internal diffusion* control is reached. Any substrate molecule diffusing into the enzyme layer is converted therein; only part of the enzyme is acting catalytically. Diffusion controlled sensors exhibit the following characteristics:

1. as long as an enzyme reserve is present, the sensitivity remains constant;
2. the sensitivity does not depend on inhibitors and pH variation;
3. the temperature is of minor influence since the activation energy of diffusion is lower than that of the enzyme reaction.

At high substrate concentration ($S^0 \gg K_M$) the enzyme reaction rate attains a limiting value, v_{\max} . Therefore the enzyme sensor signal reaches a concentration-independent value corresponding to the product concentration at the transducer surface of:

$$P^d = \frac{v_{\max} d^2}{2 D_P}.$$

Analogous equations have been established for amperometric enzyme electrodes, in which either the reaction product or a cosubstrate is converted at the electrode (Carr and Bowers, 1980, for examples see Section 2.5).

From the analysis of the coupling of enzyme reaction and mass transfer the following conclusions may be drawn for the design of biosensors.

1. The substrate concentration at which deviations from the analytically usable linear measuring range occur depends on the extent of diffusion limitation. According to the Michaelis–Menten equation, under kinetic control a linear dependence may only be expected for substrate concentrations below K_M . Under diffusion control the decrease of substrate concentration in the enzyme layer caused by slow substrate diffusion results in an extended linear range. It has to be considered, however, that for two-substrate reactions deviations from linearity may also be produced by cosubstrate consumption.
2. At low substrate concentration the sensitivity of kinetically controlled sensors increases linearly with v_{\max} . Consequently, the application of several identical enzyme layers one over the other enhances the measuring signal. When the amount of enzyme becomes sufficiently high as to provide complete substrate conversion the system passes over to diffusion control. Under these conditions a decrease of the diffusion resistance by decreasing the layer thickness results in an increased sensitivity. Nevertheless, a membrane-covered enzyme electrode is only 10 to 50% as sensitive as a bare electrode for an analogous electrode-active substance.
3. Owing to the excess of enzyme in the membrane a diffusion limited enzyme sensor has a higher functional stability than a kinetically controlled one. With the former, 2000–10 000 measurements per enzyme membrane can be performed, while kinetically controlled sensors typically permit only 200–500 measurements.
4. The response time is determined by the ratio d^2/D_P . Using fast-responding transducers, in stationary measurements a stable signal is obtained within one second up to a few minutes.

In summary, it may be concluded that optimal sensitivity and response time can be achieved by applying high enzyme activity in thin membranes.

2.4.3 Characterization of Immobilized Enzymes in Biosensors

2.4.3.1 Recovery of Enzyme Activity

In order to compare the effectiveness of different methods of immobilization it is useful to determine the portion of enzyme remaining

active after immobilization. Since the determination of the activity of the intact enzyme membrane only gives an 'apparent' value, the membrane should be resolubilized before measurement.

The remanent activity of glucose oxidase (GOD) membranes has been determined by measuring the initial rate of H_2O_2 formation with a Pt electrode polarized to +600 mV, using a known amount of resolubilized membrane in 5 mmol/l glucose solution at 37°C (Scheller et al., 1988). Thus, 70–90% of the initial activity has been found with gelatin-entrapped enzyme. The GOD membrane was solubilized in 0.05 mol/l phosphate buffer, pH 5.5, at 40°C.

After entrapment of GOD in photopolymerized polyacrylamide and resolubilization, only 22% of the initial activity was found. With GOD crosslinked to silk together with bovine serum albumin (BSA) by glutaraldehyde, only 3% enzyme remains active. The high residual activity in gelatin indicates the suitability of this matrix and the stabilizing effect of the 'active environment'. Even the rather unstable cytochrome P-450 system of liver microsomes maintains 60% of its activity when entrapped in gelatin (Schubert and Scheller, 1988a). In contrast, the activity drop of GOD in polyacrylamide indicates inactivation of the enzyme by the aggressive radical-forming reagents involved in the photopolymerization. The low residual activity determined after glutaraldehyde crosslinking is probably caused by incomplete resolubilization. Using the same method without silk as carrier, Malpiece et al. (1981) obtained a remaining GOD activity of 75%.

2.4.3.2 Effectiveness Factor

The determination of the initial rate of product formation or substrate consumption in the measuring cell using the intact membrane or the complete sensor gives a measure of the enzyme activity acting in the measuring process. By comparison with the residual activity the excess of enzyme may be estimated.

With a GOD electrode the initial rate, v_2 , of H_2O_2 accumulation has been determined in a double measuring cell containing air-saturated buffer (Fig. 27) (Scheller et al., 1983b). After glucose injection an enzyme-free electrode polarized to +600 mV indicates the rate of H_2O_2 accumulation; the other electrode is covered by a GOD membrane but not polarized so as to permit the H_2O_2 formed to diffuse into the measuring cell.

With a gelatin membrane entrapped between two dialysis membranes and containing 46 U/cm² of enzyme, the H_2O_2 formation corresponds to only 110 mU/cm², i.e., less than 1% of the initial enzyme activity (Fig. 28). This

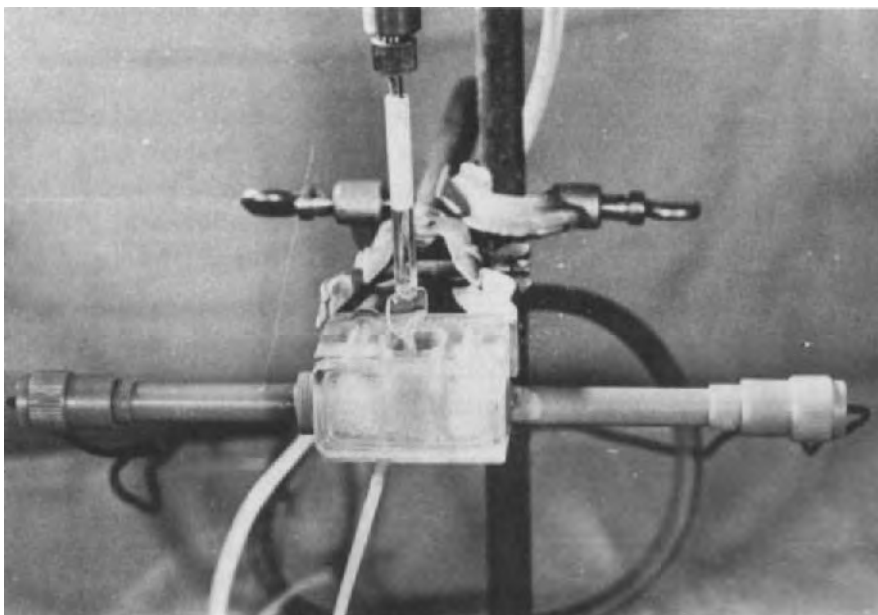


Fig. 27. Construction of a double measuring cell. For the determination of the apparent enzyme activity at the GOD electrode the rate of hydrogen peroxide accumulation in the measuring cell, v_2 , is indicated by using an enzyme-free electrode polarized to +600 mV. By contrast, when glucose is measured, the H_2O_2 diffusing to the enzyme sensor is indicated, and during the short time period of the measurement only a negligible amount of H_2O_2 diffuses into the measuring cell.

result indicates a high enzyme excess in the membrane. Consequently, the membrane is diffusion controlled. The low apparent activity may be attributed mainly to the diffusion resistance of the dialysis membrane for glucose. On the other hand, the measured activity of the membrane containing 46 mU/cm^2 already amounts to about 70% of the activity used for immobilization. This value approaches that expected for pure kinetic control of the process (Scheller et al., 1983a).

In Table 5 the apparent activities of enzymes entrapped in or covalently fixed to membranes are compared to those of enzymes directly adsorbed or fixed to electrode surfaces. It may be concluded that different immobilization procedures lead to approximately identical apparent activities. The advantage of direct attachment to the transducer surface is the low diffusion resistance of the monomolecular enzyme layer. On the other hand, enzyme membranes are more stable because of their inherent enzyme excess.

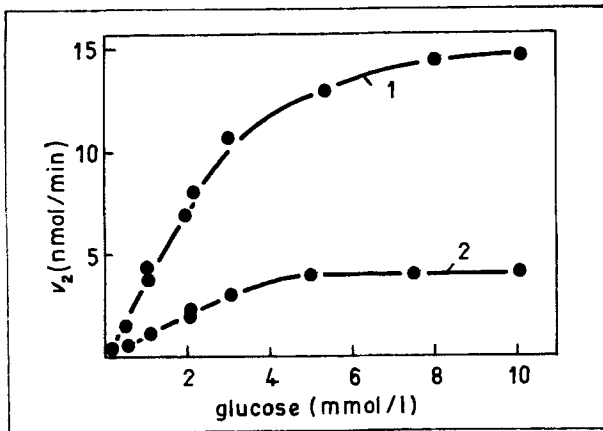


Fig. 28. Dependence of the hydrogen peroxide accumulation rate, v_2 , on substrate concentration as measured in the double measuring cell depicted in Fig. 27. v_2 is a measure of the apparent activity of GOD entrapped in a gelatin membrane. A membrane area of 0.13 mm^2 was exposed to the measuring solution. The enzyme layer was sandwiched between two dialysis membranes. Solution: 66 mmol/l phosphate buffer, pH 7.0; 25°C ; GOD loading: 1– 46 U/cm^2 , i.e., 6 U per electrode; 2– 46 mU/cm^2 , i.e. 6 mU per electrode.

2.4.3.3 Enzyme Loading Test

The variation of the enzyme loading is a means of determining the minimum amount of enzyme required for maximum sensitivity. Furthermore, this test reveals the magnitude of the enzyme reserve of diffusion controlled sensors.

Fig. 29 shows the results of a loading test of GOD entrapped in a gelatin layer of $30 \mu\text{m}$ thickness between two dialysis membranes of $15 \mu\text{m}$ thickness each. The stationary currents for 0.14 mmol/l glucose (lower part of the linear measuring range) and for 5 mmol/l glucose (saturation) increase linearly with enzyme loading from 46 mU/cm^2 to 1 U/cm^2 . At higher GOD loading a saturation value is attained. To calculate the enzyme loading factor, f_E , the following values have been used:

thickness, $d = 30 \mu\text{m}$,

Michaelis constant for glucose, $K_M = 10 \text{ mmol/l}$,

diffusion coefficient of glucose, $D_S = 1.63 \cdot 10^{-6} \text{ cm}^2$.

As is evident from Fig. 29, the transient from the linear region to saturation occurs at f_E values between 7 and 20. This agrees with the

TABLE 5

Apparent Enzyme Activity and K_M Value of Adsorbed Layers and Enzyme Membranes

Enzyme	Immobilization	Apparent enzyme activity (mU/cm ²)	Apparent K_M values		References
			soluble (mmol/l)	immob.	
GOD	gelatin entrapment	110	3.8	7.5	Scheller et al. (1988)
GOD	collagen, covalent	60–80		3.0	Thevenot et al. (1979)
GOD	cellulose acetate, covalent	340			Tsuchida and Yoda (1981)
GOD, BQ-modified ¹	cellulose acetate	>1000			Sternberg et al. (1988)
GOD	PVA entrapment	160–700			Mizutani et al. (1985)
GOD	spectral carbon, adsorbed	150–200			Hintsche and Scheller (1987)
GOD	carbon, covalent	50–170		3.1–19.1	Razumas et al. (1984)
β -Galactosidase	gelatin entrapment	1000			Pfeiffer et al. (1988)
Urease	cellulose triacetate, entrapment	3–30	2.3	2.4	Hamann (1988)
Cholesterol oxidase	collagen, crosslinked	3			Bertrand et al. (1979)
Creatinine amidohydrolase	cellulose acetate, covalent	1140	35	278	Tsuchida and Yoda (1983)
Creatine amidino-hydrolase	cellulose acetate, covalent	110	13.5	64.9	"
Sarcosine oxidase	cellulose acetate, covalent	13	6.7	2.4	"

¹BQ = benzoquinone

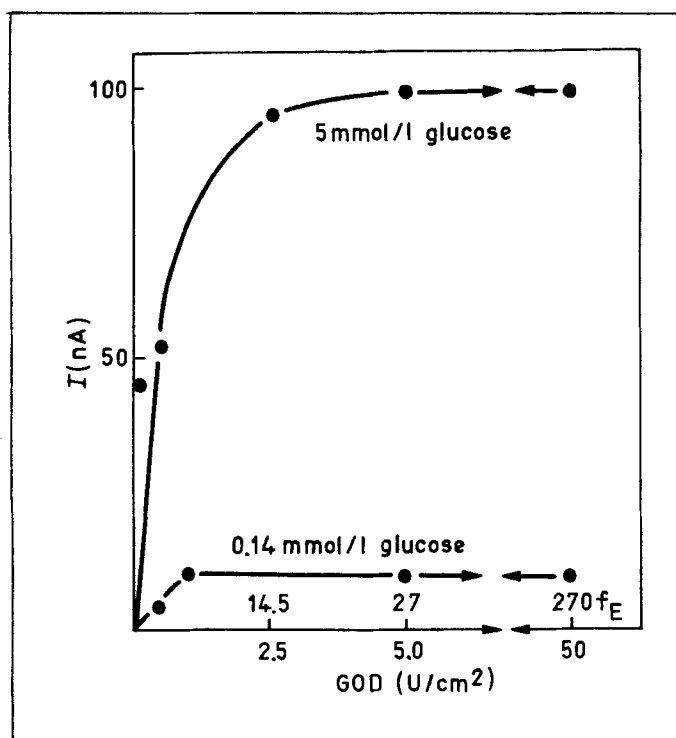


Fig. 29. Enzyme loading test of gelatin entrapped GOD as performed at pH 7 and 25°C. (Redrawn from Scheller et al., 1988).

theoretically predicted value and indicates that above 1 U/cm^2 the function of the GOD electrode is controlled by internal diffusion.

Owing to differences in the K_M values and the layer thickness, the transient from kinetic to diffusion control of different enzyme electrodes takes place at rather different enzyme activities. Gelatin-entrapped enzymes exhibit transient values of 0.17 U/cm^2 (uricase, $K_M = 17 \mu\text{mol/l}$), 16 U/cm^2 (urease, $K_M = 2 \text{ mmol/l}$) and 1.0 U/cm^2 (lactate monooxygenase, $K_M = 7.2 \text{ mmol/l}$).

2.4.3.4 Concentration Dependence of the Signal and Linear Measuring Range

The linear measuring range of biosensors extends over 2–5 decades of concentration. The lower detection limit of simple amperometric enzyme electrodes is about 100 nmol/l whereas potentiometric sensors may only be applied down to $100 \mu\text{mol/l}$. This shows that the sensitivity is effected not only by the enzyme reaction but also by the transducer.

The increase of the measuring value of the amperometric glucose electrode with increasing substrate concentration reflects the course of a Michaelis–Menten curve and reaches a concentration-independent saturation corresponding to the maximum rate, v_{\max} . The sensitivity of the GOD electrode depends on the enzyme loading (Fig. 30) (Scheller et al., 1988). The substrate concentration giving rise to the half-maximum current in air saturated solution is between 1.4 and 1.8 mmol/l glucose.

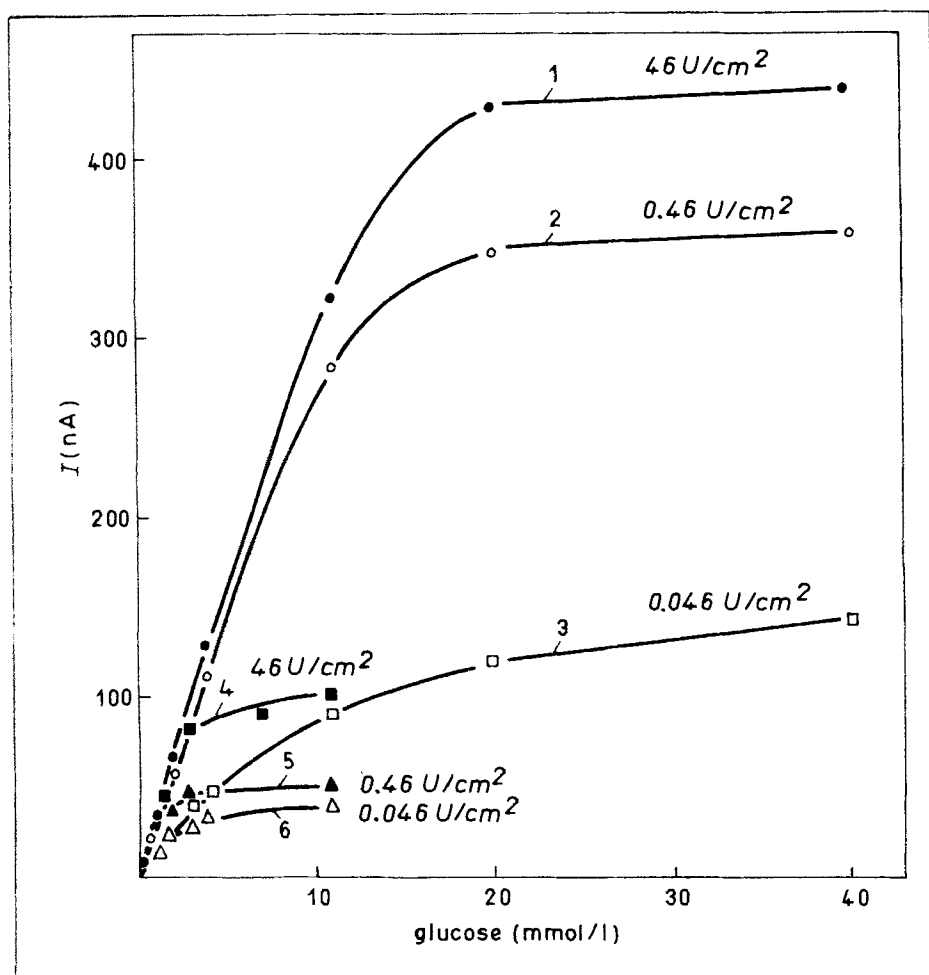


Fig. 30. Dependence of the stationary current of a GOD electrode on glucose concentration at different enzyme loadings. Electrode surface: 0.22 mm^2 ; electrode potential: +600 mV vs. Ag/AgCl; conditions as in Fig. 28; curves 1–3: oxygen-saturated solution; curves 4–6: air-saturated solution.

The linear range extends to 2 mmol/l glucose in the measuring cell. In this region, saturation of the measuring solution by oxygen increases the measuring signal by only 10%. At low glucose concentration the cosubstrate concentration (ca. 200 $\mu\text{mol/l}$ at air saturation) influences the enzyme reaction only slightly. By contrast, in the saturation region above 2 mmol/l glucose the current rises by a factor of 4.5. At the same time the linear range is extended by oxygen saturation.

At low enzyme loading the plot of the reciprocal current versus the reciprocal glucose concentration gives a straight line and thus follows the Michaelis–Menten equation (Fig. 31). From this curve an apparent $K_M(\text{glucose})$ of 7.5 mmol/l may be calculated. The apparent K_M of soluble GOD has been determined to be 3.8. mmol/l. The higher K_M of the

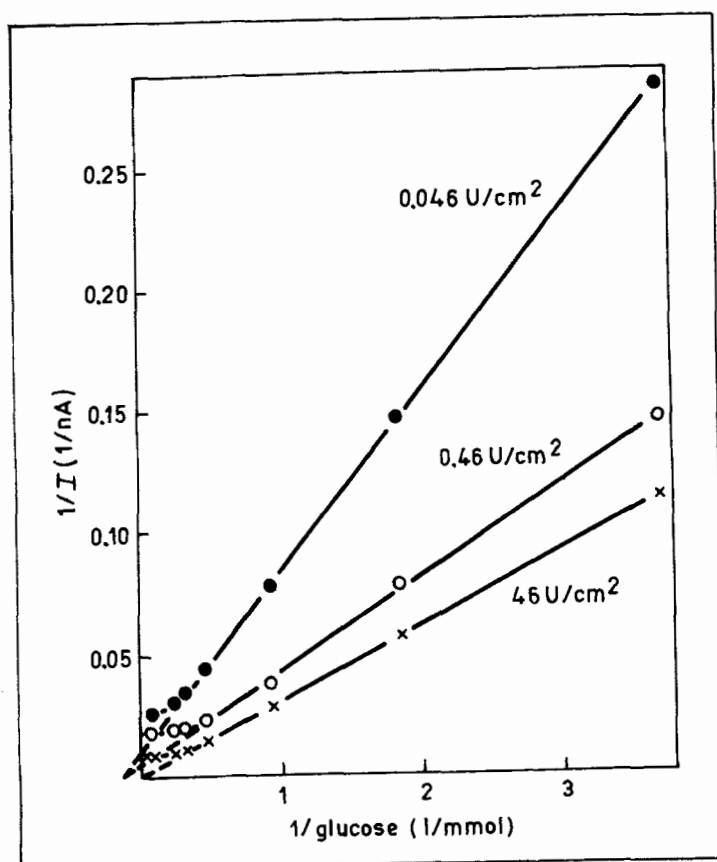


Fig. 31. Electrochemical Lineweaver–Burk plot of GOD electrodes. Conditions as in Fig. 30; air-saturated solution.

immobilized enzyme gives evidence of a superposition of diffusional and kinetic limitation.

An increase of the apparent K_M values has also been described for other enzymes immobilized by different methods (see Table 5). This enhancement of the apparent K_M enlarges the linear measuring range.

2.4.3.5 pH Dependence

With a high enzyme excess in the membrane, pH variations should have only a minor influence on the measuring process. Therefore the pH profiles in the linear measuring range and under diffusion control should be substantially less sharp than those of the respective enzyme in solution (Carr and Bowers, 1980). The results obtained with a GOD-gelatin membrane (Fig. 32) agree with this assumption (Scheller et al., 1988). With 0.14 mmol/l glucose the curve is almost as flat as that of the H_2O_2 signal. On the other hand, with 10 mmol/l a pronounced maximum is found. At this saturating concentration the signal depends on the enzyme activity and therefore distinctly on pH. The pH optimum of immobilized GOD is about 0.9 pH units more alkaline than that of the soluble enzyme. Obviously the formation of gluconic acid within the enzyme membrane causes a local pH decrease, shifting the optimum to higher pH in the solution.

Analogous pH dependences have been observed for other enzyme sensors (Carr and Bowers, 1980).

2.4.3.6 Temperature Dependence

The rate of enzyme reactions rises with temperature up to a certain optimum. Above that, the effect of thermal inactivation dominates over that of the increase of the collision frequency.

Enzyme stabilization by immobilization is frequently reflected by an increase of the temperature optimum for substrate conversion. If kinetic and diffusion control are superimposed, the higher activation energy results in a predominant acceleration of the enzyme reaction with rising temperature. Thus, the slower enhancement of the diffusion rate makes mass transfer the limiting factor. Therefore, the activation energy determined at lower temperatures is ascribed to the enzyme reaction, and that at higher temperatures to diffusion. Besides this, the temperature profile is affected by temperature-dependent conformational changes of the enzyme and decreasing solubility of the cosubstrate.

The glucose sensor with the GOD-gelatin membrane exhibits a temperature optimum of about 40°C (Scheller et al., 1988). Below the

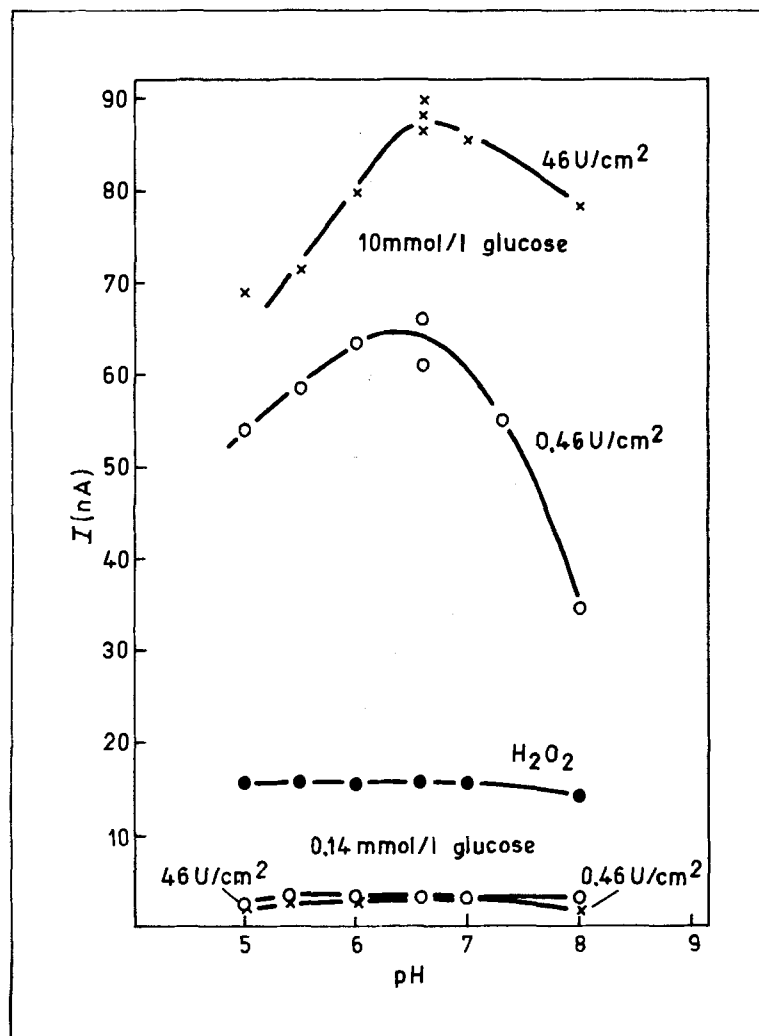


Fig. 32. pH dependence of the signal measured with a GOD electrode. Conditions as in Fig. 30.

optimum the Arrhenius plot (Fig. 33) gives parallel straight lines for different glucose concentrations and enzyme loadings. The difference between the activation energy of H_2O_2 diffusion, 33.5 kJ/mol, and that of GOD-catalyzed glucose oxidation, 25.5 kJ/mol, is probably too small to give rise to two separate linear regions. That is why purely diffusion controlled GOD electrodes are not significantly different from kinetically controlled ones with regard to activation energy.

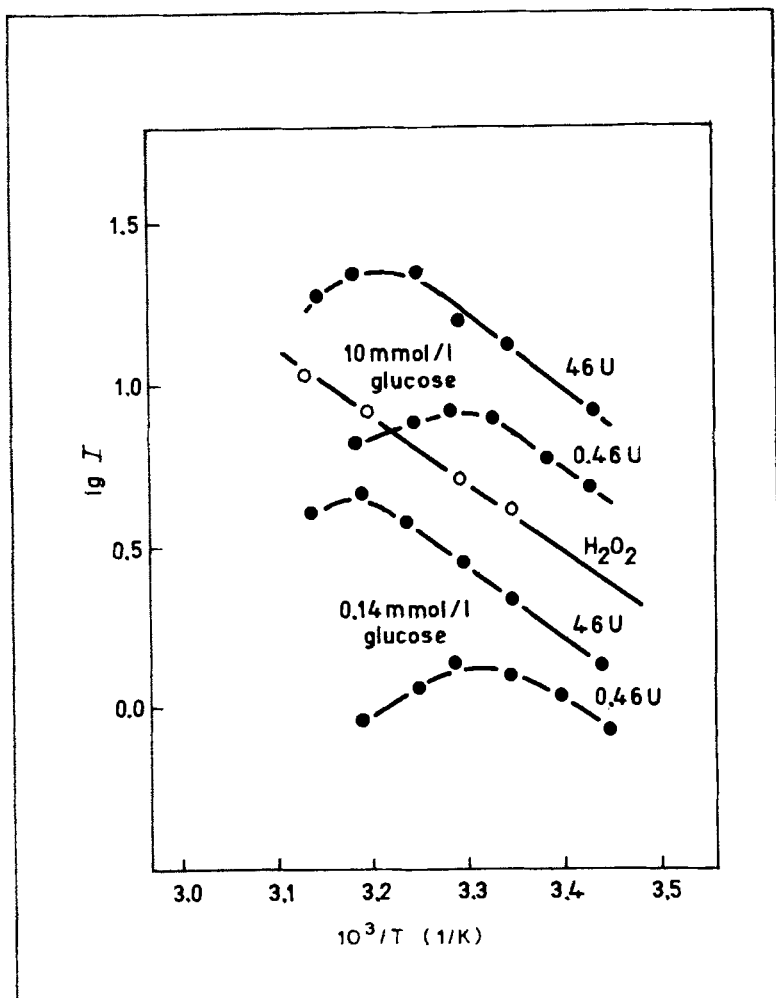


Fig. 33. Arrhenius plot of the temperature dependence of the response of a GOD electrode. Conditions as in Fig. 30.

2.5 MATHEMATICAL MODELING OF AMPEROMETRIC ENZYME ELECTRODES

2.5.1 Fundamentals of Mathematical Modeling

Since the concept of enzyme electrodes was introduced (Clark and Lyons, 1962, Updike and Hicks, 1967), numerous mathematical models of these sensors have been investigated. Because in general only the

measurement of overall processes in enzyme electrodes is possible experimentally, the use of models has been an important method for optimizing their analytical characteristics.

Various approaches have been used for the development and study of mathematical models of amperometric enzyme electrodes. Basically, one may distinguish between a physically-oriented approach that attempts to obtain a good correspondence with reality (e.g., Leyboldt and Gough, 1984) and a qualitatively oriented one that is directed at easy manipulation (e.g., Carr and Bowers, 1980; Kulys, 1981). Both approaches appear to be useful and necessary, and both have their relative limits and merits. In the present chapter the latter direction will be followed.

The models of amperometric enzyme electrodes may be classified according to the following attributes:

1. *Number of enzyme reactions modeled*

The basic idea behind enzyme electrodes is the determination of an analyte by measurement of the concentration of other, more easily measurable substances stoichiometrically related to the analyte. In simple enzyme electrodes one enzyme suffices to convert a non detectable substrate to an electrode-active product. For the determination of particular substrates the application of two or more enzymes becomes increasingly significant.

2. *Number of layers*

Usual enzyme electrodes are assemblies of several membranes placed one on top of the other. The sensitive enzyme membrane is mostly mechanically stabilized by a thin, enzyme-free dialysis membrane. Whether this arrangement is taken into account or neglected in favor of simplicity affects the quality of the model to a major extent. In sensors using more than one enzyme, the enzymes may be coimmobilized in one membrane or applied as immobilized layers in separate, sandwiched membranes. The respective models are different.

3. *Linearity or nonlinearity of the enzyme reaction*

A property that is particularly important for model formation is the linearity or nonlinearity of the reaction kinetics. Linear reaction rates permit models with parabolic differential equations having linear terms. This in turn allows the derivation of explicit solution formulas which are substantially better suited to the simulation of the sensor dynamics than numerical calculations. Since, for the enzyme elec-

trodes with which we are here concerned, direct proportionality between substrate concentration and reaction rate is assumed, linear terms may be used. With a few exceptions the application of nonlinear models leads to purely numerical methods of solution, which are much more tedious than explicit formulas.

4. *Stationary or nonstationary (rate) measurement*

The modeling of stationarily operating enzyme electrodes is a special case of nonstationary modeling. In this context, stationary means the consideration of the behaviour after infinite time (steady state), i.e., solutions have to be found for which the time derivative has to be zero. For all models considered, the existence of a unique and stable mathematical solution is assumed. Since the respective partial differential equations are linear initial boundary problems, known existence and uniqueness theorems may be used (Kamke, 1956; Fife, 1979; Özisik, 1980).

5. *Description of the situation at the boundaries*

Besides the differential equations the complete formulation of the model requires a set of initial and boundary conditions. These must reflect the situation at the interface between measuring solution and enzyme electrode membrane and between membrane and sensor. For the models considered, it is assumed that the measuring solution is perfectly mixed and contains a large amount of substrate as compared to the substrate converted in the enzyme membrane. It has been shown experimentally (Carr and Bowers, 1980) that in measuring solutions diffusion is much more rapid than in membranes. A boundary layer effect is not considered. On the sensor side all electrode-inactive substances fulfill zero flux conditions. If the model contains more than one layer the transfer between the layers may be modeled by using relations of mass conservation. The respective equations will be given in the following sections.

6. *The electrode-active substance*

According to the applied potential, an electrochemical conversion takes place at the transducer. Different potentials may therefore lead to completely different enzyme electrodes and models. In the model used, the concentration of the electrochemically converted substance is zero at the transducer.

Almost all mathematical models are identical in that

- (i) a reaction-diffusion system according to Fick's second law is used;
- (ii) the number of the spatial dimensions is reduced to one; and
- (iii) the distribution of the immobilized enzymes is assumed to be uniform.

Obviously the number of possible models is enormous. A general modeling is impossible because the type of the solution depends essentially on the attributes chosen.

In the present chapter the following notation will be used:

- (i) Capital letters symbolize the concentration of the involved substances, e.g. S , P , Y , Z , H .
- (ii) The lower case letters x and t denote the space and time coordinates.
- (iii) The membrane thickness and the enzyme reaction rate constant are denoted by d and k ; $k = v_{\max}/K_M$.
- (iv) The diffusion coefficients of the substances are denoted D_S , D_P , D_Y , etc.
- (v) The derivatives of a concentration function, e.g., the space or time derivative of $S(x, t)$, are indexed accordingly, e.g. $S_x(x, t)$ or $S_t(x, t)$. For stationary solutions, $\bar{S}(x)$, $\bar{P}(x)$, etc. is used.
- (vi) The bulk concentrations are denoted S^0 , P^0 , Y^0 , etc.

The interface between the measuring solution and the membrane corresponds to $x = 0$, the sensor side of the membrane to $x = d$. In multilayer models an additional index, i , is introduced. In all cases the modeling starts at $t = 0$.

Accordingly, the modeling of the dynamic behaviour of an electrode-inactive substance in a monoenzyme layer and with a reaction rate linear with respect to S is as follows:

$$S_t = D_S S_{xx} - kS,$$

$$S(0, t) = S^0 \quad \text{for } t > 0,$$

$$S_{x(d, t)} = 0 \quad \text{for } t > 0,$$

$$S(x, 0) = 0 \quad \text{for } 0 < x \leq d.$$

For the stationary case

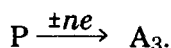
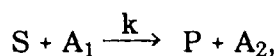
$$0 = D_S \bar{S}_{xx} - k\bar{S}$$

is valid instead of the partial differential equation.

2.5.2 The One-Layer Monoenzyme Electrode

If the given classification is used, the simplest model of an amperometric sensor is that of a stationary single-layer monoenzyme electrode with linear reaction kinetics and electrode-active product, P (Schulmeister and Scheller, 1985a).

The reaction scheme is



A_i ($i = 1, 2, 3$) denotes substances having no influence on the function of the enzyme electrode, n is the number of electrons exchanged in the electrochemical reaction.

Provided the respective concentrations are in a range that permits linearization of the reaction term, a linear approach becomes applicable for nonlinear reaction kinetics (Schulmeister and Scheller, 1985a). The model then becomes:

$$0 = D_S \bar{S}_{xx} - k\bar{S},$$

$$0 = D_P \bar{P}_{xx} + k\bar{S},$$

$$\bar{S}(0) = S^0,$$

$$\bar{S}_x(d) = 0,$$

$$\bar{P}(0) = 0,$$

$$\bar{P}(d) = 0.$$

These ordinary differential equations may easily be solved by using the formulas for undamped inhomogeneous oscillations (Kamke, 1956):

$$\bar{S}(x) = S^0 \frac{\cosh Q(d-x)}{\cosh Qd},$$

$$\bar{P}(x) = S^0 \left\{ \frac{x}{d} \left(\frac{1}{\cosh Qd} - 1 \right) + 1 \frac{\cosh Q(d-x)}{\cosh Qd} \right\} \frac{D_S}{D_P},$$

where $Q = \sqrt{k/D_S}$.

The rate method may be modeled by the following system:

$$S_t = D_S S_{xx} - kS,$$

$$P_t = D_P S_{xx} + kS,$$

$$S(0,t) = S^0, S_x(d,t) = 0 \text{ for } t > 0, S(x,0) = 0 \text{ for } 0 < x \leq d,$$

$$P(0,t) = 0, P(d,t) = 0 \text{ for } t > 0, P(x,0) = 0 \text{ for } 0 \leq x \leq d.$$

These parabolic differential equations may be solved by using a formula for inhomogeneous heat conduction problems (Özisik, 1980):

$$S(x,t) = S^0 \left\{ 1 - \frac{4}{\pi} \sum_{n=0}^{\infty} \left[\frac{(-1)^n}{2n+1} \cos \left(\frac{2n+1}{2} \pi \frac{d-x}{d} \right) \frac{k+u \exp(-(u+k)t)}{u+k} \right] \right\},$$

$$u = D_S \frac{(2n+1)^2 \pi^2}{4d^2};$$

$$\begin{aligned} P(x,t) = \frac{2k}{\pi} S^0 \sum_{m=1}^{\infty} \left(\frac{m\pi x}{d} \right) & \left\{ \frac{1-(-1)^m}{mw} [1 - \exp(-wt)] \right. \\ & + \frac{4(-1)^m}{\pi} \sum_{n=0}^{\infty} \left[\frac{(-1)^n}{(2n+1)(k+u)} \left(\frac{k(1-\exp(-wt))}{w} \right. \right. \\ & \left. \left. + \frac{u[\exp(-(k+u)t) - \exp(-wt)]}{w-k-u} \right) \frac{4m}{4m^2 - (2n+1)^2} \right] \right\}; \end{aligned}$$

$$w = D_P \frac{m^2 \pi^2}{d^2}.$$

These formulas are readily programmable on a personal computer.

Schulmeister and Scheller (1985a) applied the model to an enzyme electrode for β -D-glucose.

The current–time curve may be derived from Faraday’s law and Fick’s first law (Adam et al., 1977):

$$I(t) = n F A D_P P_x(d, t),$$

where $F = 9.65 \cdot 10^4$ C/mol is the Faraday constant and A the sensor surface area. Thus the current formula for the stationary regime is:

$$I_{st} = n F A D_P P_x(d),$$

i.e., for the case under consideration:

$$I_{st} = n F A D_P S^0 \left(\frac{1}{\cosh Qd} - 1 \right) / d.$$

The derived current formulas are series of trigonometric and exponential expressions (with negative exponent). Accordingly, the slope decays with time until a saturation is reached, which is described by the respective stationary formula. A typical curve is shown in Fig. 34.

Current–time curves may be fitted to experimental current–time data using appropriate least-squares procedures. In this way unknown sensor parameters, such as membrane thickness, d , or the kinetic rate constant, k , can be determined (Fig. 34). The quality of the parameters essentially depends on the exactness of the model and the given electrode parameters. Unique fitting of the current–time curves to experimental data is only possible with maximally three free parameters.

The described one-layer model does not account for dialysis membranes. Therefore it is useful for quantitative investigations only to a limited extent. However, it is very well suited for qualitative modeling.

2.5.3 One-Layer Bienzyme Electrodes

Three types of coupling of two enzyme reactions may be distinguished: sequential, competitive, and counteracting (cyclic). Any type has been applied for the construction of biosensors (Renneberg et al., 1986; Scheller et al., 1987c). Linear modeling leads to systems of differential equations being a generalization of those presented in Section 2.5.2. In general the same formulas may be used, albeit repeatedly.

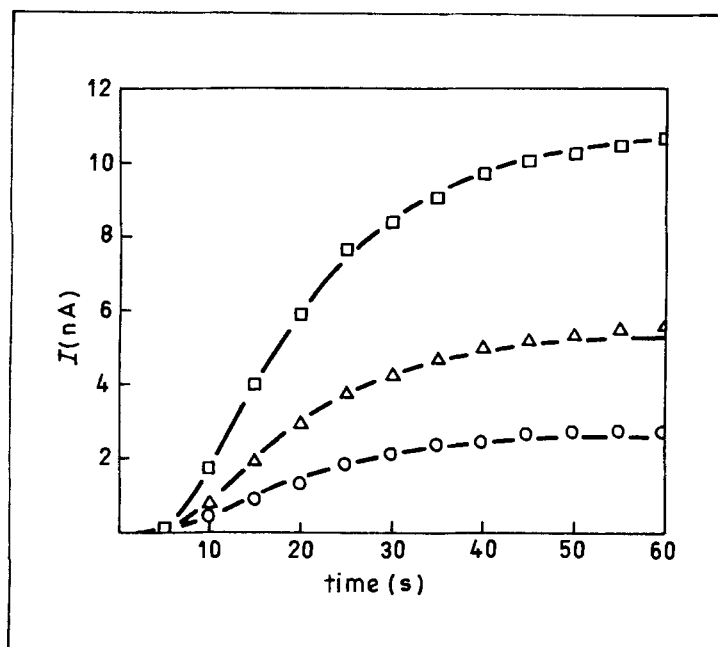
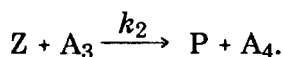
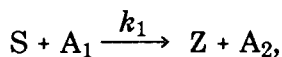


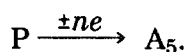
Fig. 34. Simulated current-time behavior of a GOD electrode. The electrode parameters were determined by least-squares fits of experimentally measured current-time data to be: $D_S = 1.53 \cdot 10^{-4} \text{ mm}^2/\text{s}$, $D_P = 3.56 \cdot 10^{-4} \text{ mm}^2/\text{s}$, $k = 0.95 \text{ s}^{-1}$, $d = 0.094 \text{ mm}$. Glucose concentration: ○: 0.071 mmol/l, △: 0.142 mmol/l, □: 0.285 mmol/l. (Redrawn from Schulmeister and Scheller, 1985a).

2.5.3.1 Enzyme Sequence Electrodes

In this sensor type the product, Z, of a primary enzyme reaction is converted by a second enzyme reaction to a further product, P (Pfeiffer et al., 1980; Kulys, 1981; Renneberg et al., 1982; Kulys et al., 1986a):



Z as well as P may be electroactive substances. Consider the latter case:



The mathematical model is:

$$S_t = D_S S_{xx} - k_1 S,$$

$$Z_t = D_Z Z_{xx} + k_1 S - k_2 Z,$$

$$P_t = D_P P_{xx} + k_2 Z,$$

$$S(0,t) = S^0, S_x(d,t) = 0 \text{ for } t > 0, S(x,0) = 0 \text{ for } 0 < x \leq d,$$

$$Z(0,t) = 0, Z_x(d,t) = 0 \text{ for } t > 0, Z(x,0) = 0 \text{ for } 0 < x \leq d.$$

$$P(0,t) = 0, P(d,t) = 0 \text{ for } t > 0, P(x,0) = 0 \text{ for } 0 < x \leq d.$$

with the solutions (Schulmeister and Scheller, 1985b):

$$S(x,t) = S^0 \left\{ 1 - \frac{4}{\pi} \sum_{m=0}^{\infty} \frac{\sin[(2m+1)\pi x/(2d)]}{2m+1} \cdot \frac{k_1 + u \exp[-(u+k_1)t]}{k_1 + u} \right\},$$

$$Z(x,t) = \frac{4}{\pi} k_1 S^0 \sum_{m=0}^{\infty} \frac{\sin[(2m+1)\pi x/(2d)]}{2m+1} \cdot \frac{u}{k_1 + u} \cdot \left\{ \frac{1 - \exp[-(k_2 + w)t]}{k_2 + w} \cdot \frac{\exp[-(k_1 + u)t] - \exp[-(k_2 + w)t]}{w + k_2 - u - k_1} \right\},$$

$$u = D_S \frac{(2m+1)^2 \pi^2}{4d^2},$$

$$w = D_Z \frac{(2m+1)^2 \pi^2}{4d^2},$$

$$P(x,t) = \frac{8}{\pi^2} \cdot k_1 k_2 S^0 \sum_{n=1}^{\infty} (-1)^n \sin\left(\frac{n\pi x}{d}\right) \sum_{m=0}^{\infty} \frac{(-1)^{m+1}}{2m+1} \frac{n}{n^2 - (2m+1)^2/4} \cdot \frac{u}{k_1 + u} \left\{ \frac{1}{w + k_2} \left[\frac{1 - \exp(yt)}{y} - \frac{\exp[-(k_2 + w)t] - \exp(-yt)}{y - k_2 - w} \right] - \frac{1}{w + k_2 - u - k_1} \left[\frac{\exp[-(k_1 + u)t] - \exp(-yt)}{y - k_2 - u} - \frac{\exp[-(k_2 + w)t] - \exp(-yt)}{y - k_2 - w} \right] \right\}$$

$$y = D_P \frac{n^2 \pi^2}{d^2}.$$

and the respective stationary solutions (Schulmeister and Scheller, 1985b; Kulys et al., 1986a):

$$\bar{S}(x) = S^0 \frac{\cosh Q_1 (d-x)}{\cosh Q_1 d},$$

$$\bar{Z}(x) = S^0 \frac{k_1}{D_Z(Q_2^2 - Q_1^2)} \left\{ \frac{\cosh Q_1 (d-x)}{\cosh Q_1 d} - \frac{\cosh Q_2 (d-x)}{\cosh Q_2 d} \right\},$$

$$Q_1 = \sqrt{k_1/D_S},$$

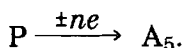
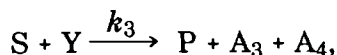
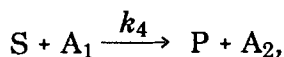
$$Q_2 = \sqrt{k_2/D_Z},$$

$$\begin{aligned} \bar{P}(x) &= \frac{k_1 k_2 S^0}{D_Z D_P (Q_2^2 - Q_1^2)} \left[\frac{1}{Q_1^2} - \frac{1}{Q_2^2} \right] \left(\frac{x}{d} - 1 \right) + \frac{\cosh Q_1 (d-x) - x/d}{Q_1^2 \cosh Q_1 d}, \\ &= \frac{\cosh Q_2 (d-x) - x/d}{Q_2^2 \cosh Q_2 d}. \end{aligned}$$

Figure 35 shows concentration profiles calculated from these equations (S — maltose, Z — glucose, P — hydrogen peroxide).

2.5.3.2 Enzyme Competition Electrodes

In this sensor type a common substrate, S, is used by two different enzymes. One of the two reactions gives the electrode-active product, P. This reaction is in the stationary state. The other reaction is started at time zero by addition of a cosubstrate, Y, which is to be measured. This reaction also consumes substrate and thus diminishes the formation of product. The resulting current decrease is utilized as the measuring signal (Pfeiffer et al., 1980; Kulys, 1981; Kulys et al., 1986a) according to:



The initial conditions of the monoenzyme electrode formulas may be used for the mathematical model (Schulmeister and Scheller, 1985b).

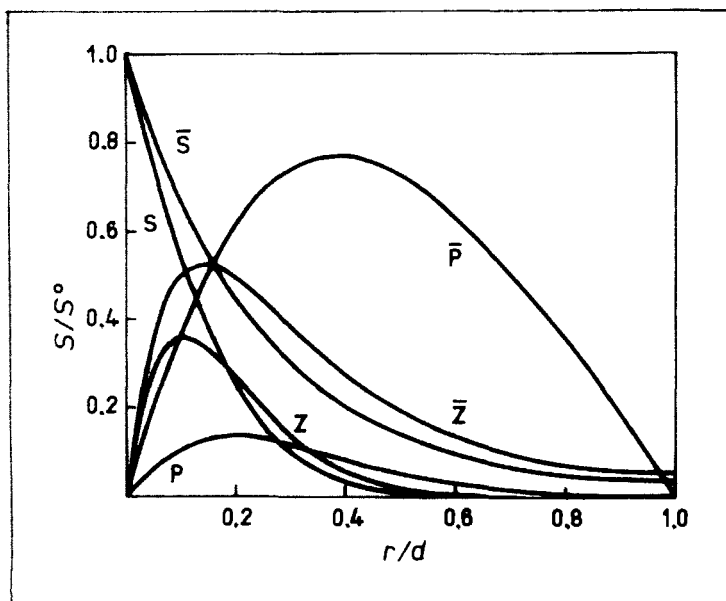


Fig. 35. Concentration profiles of a model maltose sequence electrode. Parameters: $D_S = 7.52 \cdot 10^{-5} \text{ mm}^2/\text{s}$, $D_Z = 1.53 \cdot 10^{-4} \text{ mm}^2/\text{s}$, $D_P = 2.46 D_Z$, $k_1 = 0.06 \text{ s}^{-1}$, $k_2 = 0.95 \text{ s}^{-1}$, $d = 0.141 \text{ mm}$. Profiles are given for $t = 5 \text{ s}$ and the stationary case (with bar). Maltose (S), glucose (Z), and hydrogen peroxide (P) are rendered dimensionless with d and S^0 , respectively. (Redrawn from Schulmeister and Scheller, 1985b).

$$S_t = D_S S_{xx} - k_4 S - k_3 Y$$

$$P_t = D_P P_{xx} + k_4 S$$

$$Y_t = D_Y Y_{xx} - k_3 Y$$

$$S(0,t) = S^0, S_x(d,t) = 0 \quad \text{for } t > 0,$$

$$S(x,0) = S^0 \frac{\cosh Q_4(d-x)}{\cosh Q_4 d} \quad \text{for } 0 \leq x \leq d,$$

$$P(0,t) = P^0, P(d,t) = 0 \quad \text{for } t > 0,$$

$$P(x,0) = P^0 \left(1 - \frac{x}{d}\right) + S^0 \left\{ \frac{x}{d} \left(\frac{1}{\cosh Q_4 d} - 1 \right) + 1 - \frac{\cosh Q_4(d-x)}{\cosh Q_4 d} \right\} \frac{D_s}{D_P}$$

$$\text{for } 0 \leq x \leq d,$$

$$Y(0,t) = Y^0, Y_x(d,t) = 0 \quad \text{for } t > 0,$$

$$Y(x,0) = 0 \quad \text{for } 0 \leq x \leq d.$$

If the measuring arrangement requires simultaneous addition of substrate and cosubstrate (analyte), the initial conditions for S and P are zero and other formulas are obtained (Schulmeister and Scheller, 1985b), i.e., in the case considered here:

$$S(x,t) = S^0 \frac{\cosh Q_4(d-r)}{\cosh Q_4 d} - k_3 Y^0 \frac{4}{\pi} \sum_{m=0}^{\infty} \frac{\sin[(2m+1)\pi x/(2d)]}{2m+1} \cdot \frac{v}{v+k_3} \cdot \left\{ \frac{1 - \exp[-(k_4+z)t]}{k_4+z} - \frac{\exp[-(k_3+v)t] - \exp[-(k_4+z)t]}{k_4+z-k_3-v} \right\},$$

$$Y(x,t) = Y^0 \left\{ 1 - \frac{4}{\pi} \sum_{m=0}^{\infty} \frac{\sin[(2m+1)\pi x/(2d)]}{2m+1} \frac{k_3+v \exp[-(k_3+v)t]}{k_3+v} \right\},$$

$$v = D_Y \frac{(2m+1)^2 \pi^2}{4d^2}$$

$$z = D_S \frac{(2m+1)^2 \pi^2}{4d^2},$$

$$P(x,t) = S^0 \frac{D_S}{D_P} \left\{ \frac{x}{d} \left[\frac{1}{\cosh Q_4 d} - 1 \right] - \frac{\cosh Q_4(d-x)}{\cosh Q_4 d} + 1 \right\} + P^0 \left(1 - \frac{x}{d} \right) - Y^0 k_3 k_4 \frac{8}{\pi^2} \sum_{n=1}^{\infty} \sin\left(\frac{n\pi x}{d}\right) (-1)^n \sum_{m=0}^{\infty} \frac{(-1)^{m+1}}{2m+1} \frac{n}{n^2 - (2m+1)^2/4} \cdot \frac{v}{k_3+v} \left\{ \frac{1}{k_4+z} \left[\frac{1 - \exp(-ut)}{u} - \frac{\exp[-(k_4+z)t] - \exp(-ut)}{u - k_4 - z} \right] - \frac{1}{k_4+z-k_3-v} \left[\frac{\exp[-(k_3+v)t] - \exp(-ut)}{u - k_3 - v} - \frac{\exp[-(k_4+z)t] - \exp(-ut)}{u - k_4 - z} \right] \right\},$$

$$u = D_P \frac{n^2 \pi^2}{d^2}.$$

The stationary solutions are:

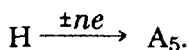
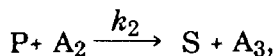
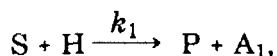
$$\bar{S}(x) = Y^0 \frac{k_3}{D_Y(Q_3^2 - Q_4^2)} \left\{ \frac{\cosh Q_3(d-x)}{\cosh Q_3 d} - \frac{\cosh Q_4(d-x)}{\cosh Q_4 d} \right\} \\ + S^0 \frac{\cosh Q_4(d-x)}{\cosh Q_4 d},$$

$$\bar{Y}(x) = Y^0 \frac{\cosh Q_3(d-x)}{\cosh Q_3 d},$$

$$\bar{P}(x) = -\frac{k_4}{D_P} \left\{ \frac{S^0}{Q_4^2} \left[\frac{\cosh Q_4(d-x)}{\cosh Q_4 d} + \left(1 - \frac{1}{\cosh Q_4 d} \right) \frac{x}{d} - 1 \right] \right. \\ + \frac{k_3 Y^0}{D_S(Q_3^2 - Q_4^2)} \left[\frac{\cosh Q_3(d-x)}{Q_3^2 \cosh Q_3 d} - \frac{\cosh Q_4(d-x)}{Q_4^2 \cosh Q_4 d} \right. \\ \left. \left. + \frac{x}{d} \left(\frac{\cosh Q_3 d - 1}{Q_3^2 \cosh Q_3 d} - \frac{\cosh Q_4 d - 1}{Q_4^2 \cosh Q_4 d} \right) - \left(\frac{1}{Q_3^2} - \frac{1}{Q_4^2} \right) \right] \right\} + P^0 \left(1 - \frac{x}{d} \right)$$

2.5.3.3 Cyclic Enzyme Reactions

The basic principle of cycling sensors is the use of an enzyme pair which continuously cycles the substrate, S, and therefore causes a relatively large concentration change of an electrode-active cosubstrate, H (Scheller et al., 1985b; Schubert et al., 1985a, 1986b; Mizutani et al., 1985; Kulys et al., 1986a):



The relevant mathematical model can be written as follows:

$$S_t = D_S S_{xx} - k_1 S + k_2 P,$$

$$P_t = D_P P_{xx} + k_1 S - k_2 P,$$

$$H_t = D_H H_{xx} - k_1 S,$$

$$S(0,t) = S^0, S_x(d,t) = 0 \text{ for } t > 0, S(x,0) = 0 \text{ for } 0 \leq x \leq d,$$

$$P(0,t) = 0, P_x(d,t) = 0 \text{ for } t > 0, P(x,0) = 0 \text{ for } 0 \leq x \leq d.$$

$$H(0,t) = H^0, H(d,t) = 0 \text{ for } t > 0, H(x,0) = H^0 \left(1 - \frac{x}{d}\right) \text{ for } 0 \leq x \leq d.$$

In the case under consideration stationary initial conditions are assumed for the cosubstrate, H. This corresponds to an existing arrangement (Scheller et al., 1985b; Mizutani et al., 1985).

An explicit solution of the differential equations requires the assumption

$$D_S = D_P = D.$$

This is only a minor restriction, since the recycled substrates are usually similar. The following formulas are obtained (Schulmeister, 1987b):

$$S(x,t) = S^0 \frac{4}{\pi} \sum_{n=0}^{\infty} \frac{\sin[(2n+1)\pi x/(2d)]}{2n+1} \left\{ \frac{k_2 + v}{k + v} [1 - \exp(-(k+v)t)] \right.$$

$$\left. \left\{ -\frac{k_2}{k} [\exp(-vt) - \exp(-(k+v)t)] \right\}, \right.$$

$$P(x,t) = S^0 \left[1 - \frac{4}{\pi} \sum_n \frac{\sin [(2n+1)\pi x/(2d)]}{2n+1} \exp(-vt) \right] - S(x,t),$$

$$v = D \frac{(2n+1)^2 \pi^2}{4d^2},$$

$$k = k_1 + k_2,$$

$$H(x,t) = H^0 \left(1 - \frac{x}{d}\right) - \frac{8}{\pi^2} k_1 S^0 \sum_{m=1}^{\infty} \left\{ \sin \left(\frac{m\pi(x-d)}{d} \right) \sum_{n=0}^{\infty} \frac{(-1)^n}{2n+1} \right.$$

$$\left. \cdot \frac{m}{m^2 - (2n+1)^2/4} \right.$$

$$\left[\frac{k_2 + v}{k + v} \left(\frac{1 - \exp(-wt)}{w} - \frac{\exp(-(k + v)t) - \exp(-wt)}{w - k - v} \right) - \frac{k_2}{k} \left(\frac{\exp(-vt) - \exp(-wt)}{w - v} - \frac{\exp(-(k + v)t) - \exp(-wt)}{w - k - v} \right) \right] \Bigg\},$$

$$w = D_H \frac{m^2 \pi^2}{d^2}.$$

The stationary solutions are:

$$\bar{S}(x) = S^0 \left(Q_1^2 \frac{\cosh Q(d-x)}{\cosh Qd} + Q_2^2 \right) / Q^2,$$

$$\bar{P}(x) = S^0 - \bar{S}(x),$$

$$\bar{H}(x) = H^0 \left(1 - \frac{x}{d} \right) + S^0 \frac{k_1}{D_H Q^2} \left[\frac{Q_1^2}{Q^2} \frac{\cosh Q(d-x) - x/d}{\cosh Qd} + \frac{Q_2^2}{2} x(x-d) - \frac{Q_1^2}{Q^2} \frac{(d-x)}{d} \right],$$

where $Q_1 = \sqrt{k_1/D}$, $Q_2 = \sqrt{k_2/D}$, $Q = \sqrt{k/D}$.

The concentration profile typical for the diffusion of the substrate and the formation of the product up to a stationary level is shown in Fig. 36.

The cyclic reaction provides a significantly higher cosubstrate conversion than in the case of a monoenzyme arrangement ($k_2 = 0$). The signal amplification thus obtained may shift the measuring range of the enzyme electrode by several orders of magnitude to lower analyte concentrations. The amplification factor, G , can be described as:

$$G = \frac{\frac{k_1}{Q^2} \left(\frac{k_1}{Q^2 \cosh Qd} - \frac{k_1}{Q^2} - \frac{k_2 d^2}{2} \right)}{D^2 \left(\frac{1}{\cosh Q_1 d} - 1 \right)}$$

For high enzyme loading ($Q_1 d, Qd \gg 1$) this may be approximated by:

$$G \approx \frac{k_1 k_2 d^2}{2 D k}.$$

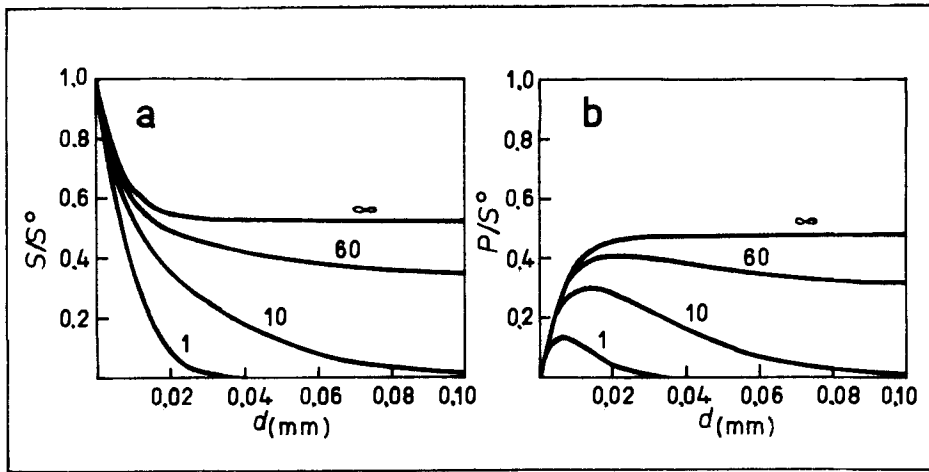


Fig. 36. Concentration profiles of a cyclic lactate-sensing electrode. Parameters: $k_1 = 1.0 \text{ s}^{-1}$, $k_2 = 1.1 \text{ s}^{-1}$, $D = 9.0 \cdot 10^{-5} \text{ mm}^2/\text{s}$, $d = 0.1 \text{ mm}$, $n = 4$. Profiles of lactate (a) and pyruvate (b) are given for $t = 1, 10, 60 \text{ s}$ and the stationary case. Lactate and pyruvate are rendered dimensionless with S^0 . (Redrawn from Schulmeister, 1987b).

2.5.4 Multilayer Enzyme Electrodes

Compared with the derivations described so far, models with more than one layer require mathematically completely different approaches. The respective systems of differential equations either have to be solved numerically (Smith, 1965; Lasia, 1983; Bergel and Comtat, 1984) or a new approach must be found. The mathematical problem is that the boundary conditions are not known for each layer. For the interfaces between the different membranes only mass conservation relations are known rather than boundary values and no-flux conditions. They may be written:

$$H^i(d_i, t_j) = H^{i+1}(0, t_j),$$

$$D_i H_x(d_i, t_j) = D_{i+1} H_x(0, t_j).$$

D_i and D_{i+1} are the diffusion coefficients of the substrate under consideration, H , in the layer i and $i + 1$, respectively. Each of the l layers is modeled separately, the layer thickness being d_i ($i = 1, \dots, l$). The equations of mass conservation are considered for fixed times, t_j ($j = 1, 2, \dots$). Then the basic idea of the multilayer approach may be described

as follows (Schulmeister, 1987a). It is known from the formula used above that with linear initial and boundary conditions the solution for a one-layer model is a multilinear function of the parameters of these initial and boundary conditions. These parameters are exactly the unknown values $H^i(0, t_1)$, $H^i(d_i, t_1)$, $H^i(0, t_2)$, and $H^i(d_{i+1}, t_2)$, where $t_2 - t_1$ is the time-step. Therefore the solution $H(x, t)$ can be determined for each layer and the space derivative at the boundary can be formed.

When the values so obtained are inserted into the mass conservation equations, a system of linear equations with respect to the desired parameters is obtained. The solution of this system is therefore the calculation of the concentration profile for the time considered, t_2 . Since for this calculation the values of the concentration profile at t_1 must be known, a successive calculation from one concentration profile to the other is necessary. Since, furthermore, the use of linear boundary and initial conditions is only an approximation, calculation has to be performed in small steps with respect to both time and space. In other words, if a sufficient refinement of the layer arrangement is chosen (by partition of the given layers) and the time steps are sufficiently small, successive calculation of concentration profiles is possible with high accuracy. This algorithm has been published and illustrated by case studies (Schulmeister, 1987a). An example is given in Fig. 37.

2.5.5 Conclusions

The linear approach described here is expandable to multienzyme electrodes as well as multilayer electrodes. At least for the stationary case, multilayer models of bienzyme electrodes may be easily treated, too. The whole system is readily adaptable to potentiometric electrodes (Carr and Bowers, 1980). It must be noted, however, that the superiority over purely numerical solution procedures decreases with increasing number of enzyme species and in the multilayer model. The advantage in calculation speed using the sum formulas described (e.g., in Section 2.5.2) amounts to about two orders of magnitude. With multilayer electrodes and formulas containing double and triple sums it is reduced to one order of magnitude.

In many cases the application of linear models is not possible, namely if:

- (i) the enzyme kinetics depend on more than one substrate; and
- (ii) the concentration range to be studied does not permit approximation of the reaction rate by an expression linearly depending on the substrate concentration.

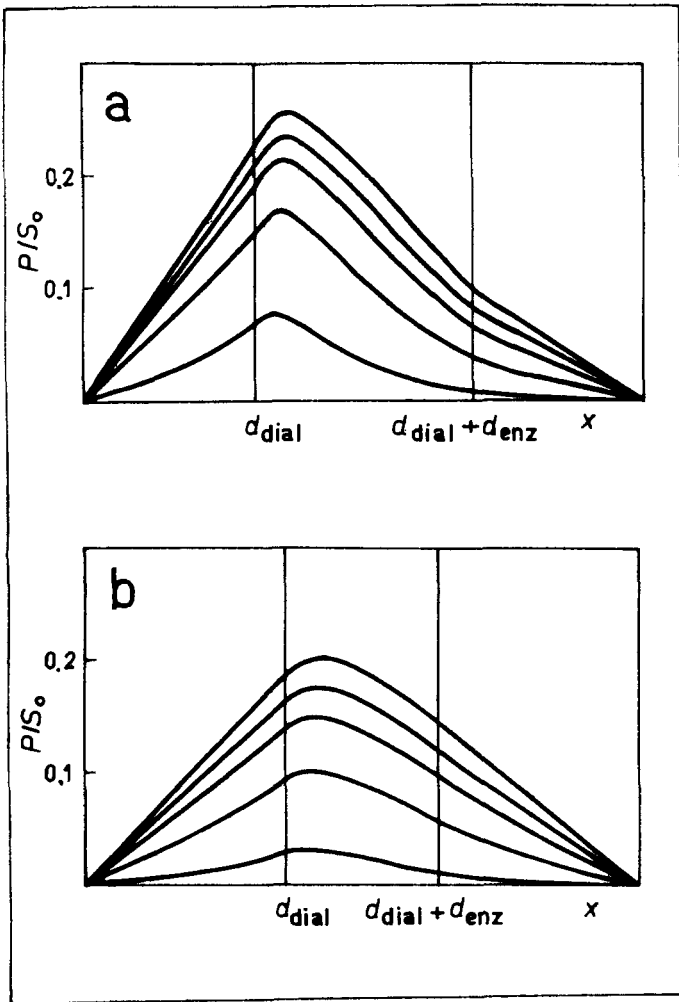


Fig. 37. Calculated concentration profiles for the product, P , in two three-layer models. In both cases an enzyme membrane of thickness d_{enz} was stabilized by two dialysis membranes of thickness d_{dial} . The profiles were obtained for $t = 1.5, 3, 4.5$, and 6 s and the stationary case. Parameter values are: (a) $k = 6.40 \text{ s}^{-1}$, $D_{S,1} = 1.18 \text{ mm}^2/\text{s}$, $D_{S,2} = 1.98 \text{ mm}^2/\text{s}$, $d_{enz} = 0.038 \text{ mm}$, $d_{dial} = 0.03 \text{ mm}$; (b) $k = 3.29 \text{ s}^{-1}$, $D_{S,1} = 1.30 \text{ mm}^2/\text{s}$, $D_{S,2} = D_{S,1}$, $d_{enz} = 0.022 \text{ mm}$, $d_{dial} = 0.029 \text{ mm}$. In each layer the diffusion coefficient of the product, D_P , equals $2.46 D_S$. (Redrawn from Schulmeister, 1987a).

Various methods have been described for the numerical realization of the respective model (Smith, 1965; Lasia, 1983). Since the appropriate boundary and initial conditions can also be employed with nonlinear reaction rates, the resulting system of parabolic differential equations

may be treated by the relatively simple line method using differences of the second order. The transformation into 100 discrete variables will usually be sufficient.

For special cases, approximate current formulas can also be derived from nonlinear models. In spite of the necessary restrictions these formulas satisfy most requirements (Carr and Bowers, 1980).

Chapter 3

Metabolism Sensors

Of all types of biosensors, metabolism sensors based on the molecular analyte recognition and conversion have been most intensively studied. According to the degree of integration of the biocomponents they can be classified into monoenzyme sensors, biosensors using coupled enzyme reactions, organelle, microbial, and tissue-based sensors. The sequence of the following sections corresponds to this classification.

3.1 MONOENZYME SENSORS

This sensor type initiated biosensor research and has reached the highest state of practical application. The enzyme sensors described and used up to now have been mainly based on oxidoreductases and hydrolases.

3.1.1 Glucose Sensors

The determination of glucose is one of the most frequently performed routine analyses in clinical chemistry as well as in the microbiological and food industries. Here, the application of glucose electrodes appears to be the method of choice. Moreover, in combination with other enzymes, glucose oxidase sensors are applicable to the measurement of di- and polysaccharides and amylase and cellulase activity, which is required in many biotechnological processes. This versatility explains, why numerous researchers worldwide are concerned with the development and optimization of glucose sensors.

With glucose sensors the different coupling variants of immobilized enzymes with signal transducers may be very well demonstrated. The variety of constructions result from the specific requirements and limits as well as the different optimal operating conditions of the sensor

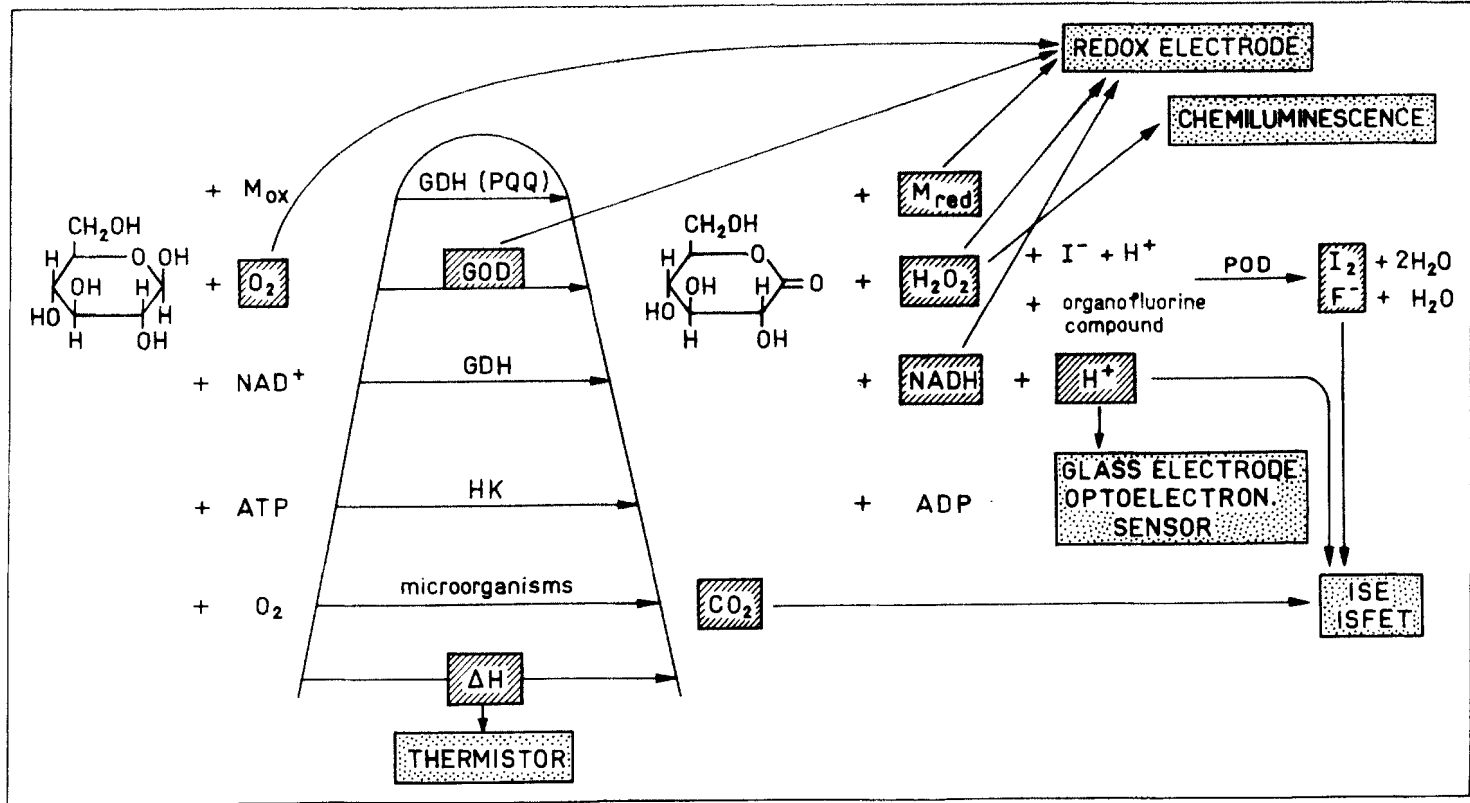


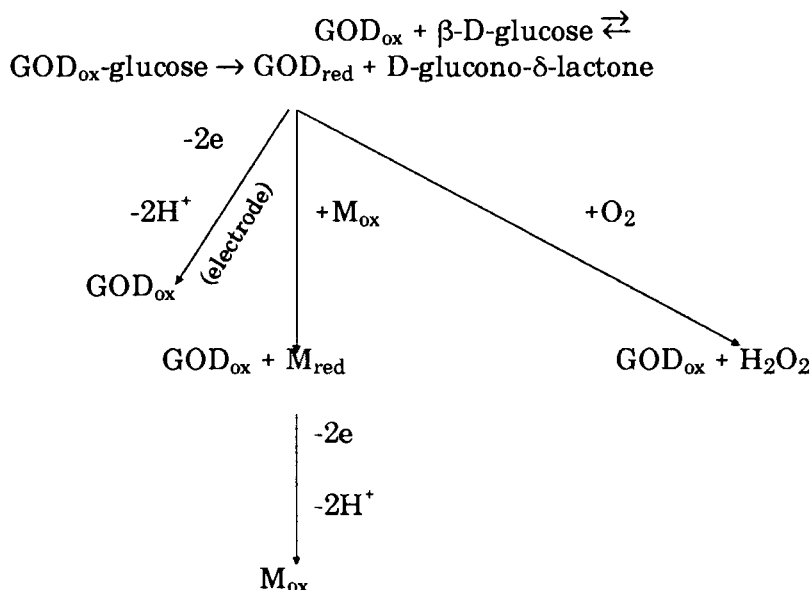
Fig. 38. Variants of glucose conversion and indication of the resulting reaction effects using biosensors. GOD: glucose oxidase, GDH: glucose dehydrogenase, GDH(PQQ): quinoprotein glucose dehydrogenase, HK: hexokinase, M_{ox} : oxidized mediator, M_{red} : reduced mediator.

verting enzymes and their use in enzyme reactors as precursors of biosensors will be discussed first, and thereafter the three generations of glucose sensors will be described.

In enzyme sensors for glucose the conversion of glucose by glucose oxidase has been mainly applied. Only a few sensors contain NADH-dependent glucose dehydrogenase, quinoprotein glucose dehydrogenase, or hexokinase (Fig. 38), or use additionally coupled horseradish peroxidase, gluconolactonase, catalase, or glucose isomerase. Microbial glucose sensors utilize the oxygen consumption accompanying oxidation of glucose to CO_2 (see Section 3.3). Furthermore, the glucose-binding glycoprotein concanavalin A has been employed in glucose sensors.

Glucose-Converting Enzymes

Glucose oxidase (GOD, EC 1.1.3.4) isolated from *Aspergillus niger* (MW 160 000) or *Penicillium notatum* (MW 152 000) is a glycoprotein consisting of two identical subunits which are connected by disulfide bridges. Each subunit contains one molecule of flavin adenine dinucleotide (FAD) as a prosthetic group. GOD catalyzes the oxidation of β -D-glucose to D-glucono- δ -lactone in the presence of an electron acceptor. As shown in the simplified reaction scheme, there are different ways to reoxidize the reduced enzyme:

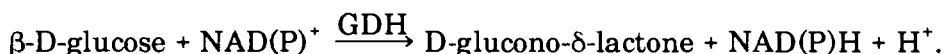


where GOD_{ox} = oxidized form of GOD; GOD_{red} = reduced form of GOD; M_{ox} = oxidized form of mediator; M_{red} = reduced form of mediator.

In the simplest case ambient oxygen is reduced to H_2O_2 . Other electron acceptors, such as hexacyanoferrate(III), may be substituted for oxygen. If the enzyme is adsorbed onto the electrode, direct electron transfer from the prosthetic group becomes feasible.

GOD is characterized by a high functional stability. Experimental data indicate a maximum stability of 10^7 cycles (Bourdillon et al., 1982). The enzyme is specific for β -D-glucose, α -D-glucose is not oxidized. This is important for the coupling of α -D-glucose-forming disaccharidase reactions. The K_M values for glucose of 9.6 mmol/l (*P. notatum*) and 33 mmol/l (*A. niger*) together with an activity up to 220 U/mg are sufficient for use in enzyme electrodes.

Selective determination of β -D-glucose has also been performed by using glucose dehydrogenase (GDH, EC 1.1.1.47):



In contrast to GOD, the cofactor of GDH, NAD^+ with EC 1.1.1.118 and NADP^+ with EC 1.1.1.119, cannot be replaced by other mediators nor by oxygen. GDH is prepared from liver or bacteria, e.g. *Bacillus megaterium*. The tetrameric molecule has a molecular weight of 118 000 and dissociates at pH 9 into identical subunits. The K_M for glucose, 47.5 mmol/l (GDH from *B. megaterium*), is in a range that is favorable for enzyme electrodes ($K_M(\text{NAD}^+) = 4.5$ mmol/l). The specific activity of GDH is similar to that of GOD.

In addition to pyridine nucleotide-dependent glucose dehydrogenases a GDH containing PQQ (2,7,9-tricarboxy-1H-pyrrolo[2,3]quinoline-4,5-dione) as prosthetic group has been isolated. This NADH-independent enzyme (EC 1.1.99.17) from *Bacterium anitratum* has a molecular weight of 86 000 and a specific activity of 3000 U/mg. This corresponds to a turnover number of $320\,000\text{ min}^{-1}$, i.e., 30-fold higher than that of GOD. GDH(PQQ) catalyzes glucose oxidation in the presence of artificial electron acceptors like NMP^+ and ferricinium, but not with O_2 , FAD, or cytochrome c. The high specific activity and the high K_M value for glucose, 11.5 mmol/l, make the enzyme well suited for enzyme electrodes (D'Costa et al., 1986; Higgins et al., 1987).

ATP-dependent *hexokinase* (HK, EC 2.7.1.1) converts both glucose anomers. Its K_M for glucose is 10 mmol/l. The tetrameric protein (MW 100 000) contains eight SH-groups per molecule, of which four are essential for the enzyme's function. Owing to the high specificity of HK, glucose determination by means of this enzyme has become the inter-

national reference method. The specific activity of yeast hexokinase of 140 U/mg suffices for application in biosensors.

3.1.1.1 Analytical Enzyme Reactors

For reasons of economy, application of enzymes as immobilized, i.e. reusable, preparations in clinical chemistry and process control has become increasingly common. Three basic types of analytical enzyme reactor have been described (Mottola, 1983):

1. In *packed bed reactors* the enzyme-catalyzed reaction is carried out in a column of 100 μl –10 ml volume. The column is filled with tiny particles bearing the immobilized enzyme. The continuously formed reaction product is indicated colorimetrically or electrochemically. Enzyme carrier materials with advantageous flow behavior are porous glass with pores of a defined size, organic polymers, like nylon powder, and inorganic polymers.
2. In *open tubular reactors* the enzyme is attached in a monolayer to the inner walls of glass or nylon tubing. Since the loading density is relatively low, for a high degree of substrate conversion a considerable reactor length (up to 4 m) may be necessary. Such reactors exhibit better flow characteristics than packed bed reactors and permit a measuring frequency up to 200/h.
3. In *single bead string reactors* the enzyme is bound both to the reactor wall and to a carrier within the reactor. Compared with open tubular reactors this reactor type provides a higher conversion at lower sample mixing.

All of these reactor types have been used for glucose determination. GOD-packed bed reactors combined with colorimetric indication are predominant. In many cases a horseradish peroxidase reactor performing a chromogenic reaction has been coupled sequentially (Gorton and Ögren, 1981). With open tubular reactors good results have been obtained using coimmobilized hexokinase and glucose-6-phosphate dehydrogenase combined with spectrophotometric NADPH indication. This arrangement is employed in the SMAC system (Technicon Co., USA), providing a sampling frequency of 150/h and an operational stability of 1 month. Technicon AAI analyzers use GOD immobilized on the inner wall of nylon tubing. A chromogenic reaction catalyzed by soluble peroxidase has been employed for colorimetric indication (Endo et al., 1979).

The combination of an enzyme reactor with direct electrochemical product indication provides a relatively simple flow-through setup.

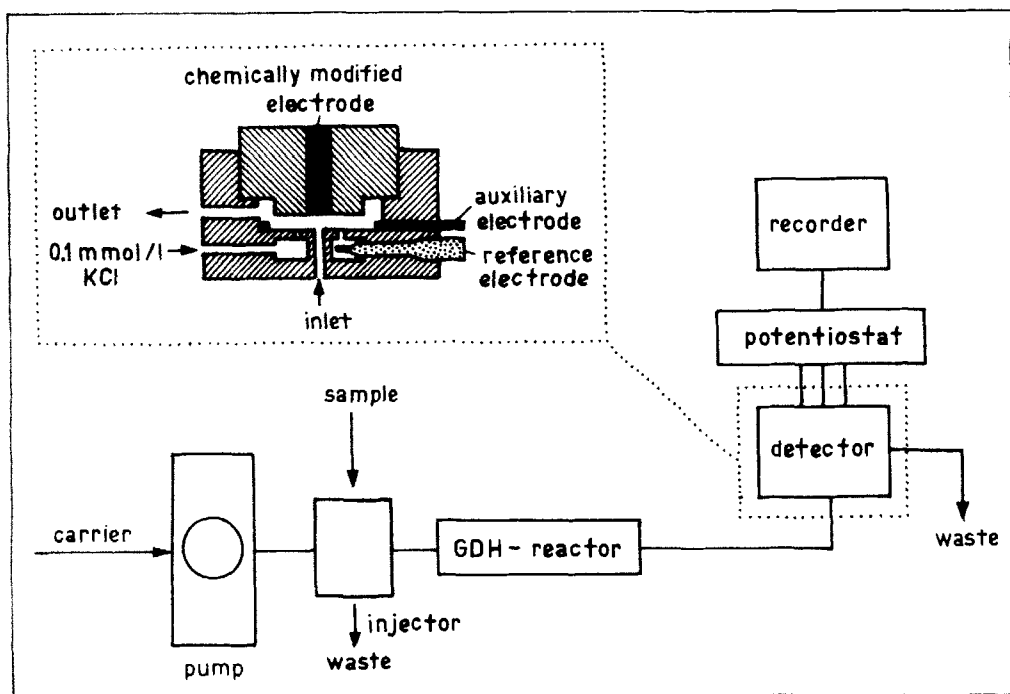


Fig. 39. Flow injection analysis (FIA) system for the determination of glucose involving a GDH reactor and a chemically modified electrode for NADH measurement. (Redrawn from Appelqvist et al., 1985).

Thus, for glucose measurement excellent parameters such as a coefficient of variation (relative standard deviation, CV) of 0.2–0.6%, have been achieved by using a flow injection analysis (FIA) device combined with a GDH reactor and an electrode modified for NADH indication (Fig. 39) (Appelqvist et al., 1985).

In flow-through enzyme thermistors the immobilized enzyme is also contained in packed bed reactors. For glucose determination with these devices, GOD has been immobilized both separately and together with catalase (Danielsson et al., 1977). In the latter system the splitting of H_2O_2 substantially enhances the overall reaction enthalpy (see Table 2), thus lowering the detection limit from 0.01 to 0.002 mmol/l glucose. The linear measuring range extends up to 1 mmol/l. Using a hexokinase reactor, the upper limit of linearity has been shifted to 25 mmol/l (Bowers and Carr, 1976), and 30–40 serum samples diluted 50–100 fold have been determined with high precision (CV = 0.6%). A GOD thermistor has been adapted to continuous glucose measurement by combination with an automatic sampling system (Danielsson et al., 1981).

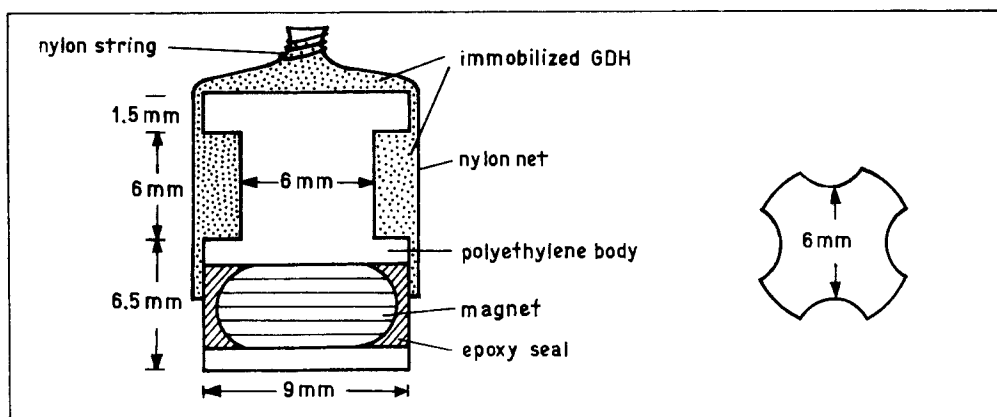


Fig. 40. Cross section of an enzyme stirrer containing immobilized GDH. (Redrawn from Kuan and Guilbault, 1977).

Kuan and Guilbault (1977) immobilized GDH on the surface of a stirrer and registered the liberated NADH fluorimetrically. The 'enzyme stirrer' (Fig. 40) was stable for two months and permitted 500 analyses of glucose in plasma to be performed.

3.1.1.2 Enzyme Membrane Sensors for Glucose

The development of enzyme membrane sensors for glucose began with the first enzyme electrode constructed by Clark. The sensors developed since differ with respect to the biological component and, even more, with the transducer type used. A common element to all of them is that the biocomponent is fixed in front of the transducer by a semi-permeable membrane.

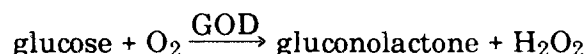
1. Potentiometric Glucose Sensors

The communication by Clark and Lyons (1962) of the first amperometric enzyme sensor already pointed to the possibility of coupling a glucose oxidase layer to a pH glass electrode. Nilsson et al. (1973) were the first to use this principle. Owing to the large response time required to reach a stationary state after substrate addition (about 10 min), the sensitivity to buffer capacity alteration, and the logarithmic concentration dependence for glucose (1–10 mmol/l), this type of sensor has not found general acceptance. These analytical characteristics are based on the fact that in the neutral region the spontaneous gluconolactone hydrolysis generating the indicated H^+ ions is so slow (half life, 3.5 min at pH 7) that almost no measuring signal is found, even at very low

buffering background (Hanazato et al., 1988). Therefore the sensitivity of the sensor is set by the content of gluconolactonase, a common constituent of commercial GOD preparations.

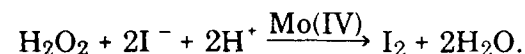
Potentiometric glucose sensors based on GOD and GOD + catalase immobilized on platinum netting or platinum wire have been described by Wingard et al. (1982). A potential change, the logarithm of which is linearly related to glucose concentration, is likely to be formed by the O_2/H_2O_2 redox couple. The authors discussed the *in vivo* application of this sensor. For this purpose, however, the measuring range, up to 8.33 mmol/l, is too narrow. Furthermore, interferences by redox active substances have not been excluded.

Several authors investigated ion selective electrodes incorporating GOD coupled with oxidative reactions catalyzed by horseradish peroxidase (HRP). Thus, glucose has been determined by measuring iodide concentration at an iodide sensitive electrode (Nagy et al., 1973) according to:



The enzymes have been both physically entrapped in polyacrylamide on nylon netting and chemically bound to polyacrylic acid derivatives; both preparations exhibited large measuring times. Improvement of the system in favour of the response time diminished the sensitivity of the sensor. The authors reported a response time between 77 and 235 s and a sensitivity of 40 mV per concentration decade. Besides the low selectivity of the iodide sensitive electrode (thiocyanate, sulfide, cyanide, and silver(I) ions interfere), disturbances by other HRP substrates, e.g. uric acid, ascorbic acid, and Fe(II) ions, restrict the applicability of the method.

Al-Hitti et al. (1984) compared the GOD–HRP sequence with a system in which HRP has been replaced by ammonium molybdate:

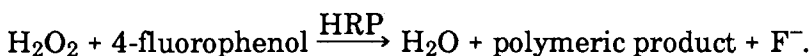


The enzymes are immobilized in poly(vinyl chloride) as follows: 50 mg GOD (6500 U) and 18 mg HRP (6000 U) are mixed with 20 mg PVC and 6 ml tetrahydrofuran; 40 mg dioctylphenyl phosphate are added as

plasticizer. A flexible membrane of 230–250 μm thickness is formed.

A sensitivity of 74 mV/decade was obtained with this sensor. With HRP the sensor was stable for 7 days, and with Mo(IV) it was stable for 3 days. The response time was between 2 and 12 min, the measuring time between 35 and 70 min. An increase of the stability to 14 days by crosslinking with glutaraldehyde and bovine serum albumin (BSA) was accompanied by a sensitivity drop to 50% (43 mV/decade).

Ho and Wu (1985) developed a potentiometric glucose sensor based on the reaction of H_2O_2 formed in the GOD reaction with an organic fluorine compound in the presence of HRP:



The fluoride formed was indicated at a fluoride sensitive electrode. The substrate, 4-fluorophenol, has a high reaction rate and a favorable diffusion behavior. The authors observed no interferences by ascorbic acid or uric acid; glutathione interfered above 0.87 mg/ml. When immobilized by glutaraldehyde crosslinking with BSA, the enzymes were stable for 30 days.

GDH immobilized in polyacrylamide gel on the surface of a platinum net has been used by Chen and Liu (1977) for potentiometric glucose determination. At the optimal NAD^+ concentration of 0.015 mmol/l and 25°C the sensitivity was 15 mV/decade between 0.1 and 5 mmol/l glucose. Addition of ferricyanide did not change these values, i.e., this substance was not active as an electron mediator.

2. Amperometric Glucose Sensors

The great number of patents and publications and of available commercial analyzers indicate the leading position of amperometric glucose sensors. These sensors allow a simple measuring regime and make use of the advantages of Faradaic electrode processes, such as independence of buffer capacity, linear concentration dependence, and high sensitivity in substrate measurement.

The cosubstrate, oxygen, and the product, hydrogen peroxide, of the GOD-catalyzed glucose oxidation are electrochemically active. Therefore, gas sensitive and redox electrodes can be used as indicator electrodes (see Fig. 38).

(i) Registration of Cosubstrate Consumption. The natural cosubstrate of GOD is atmospheric oxygen. In glucose determination the difference

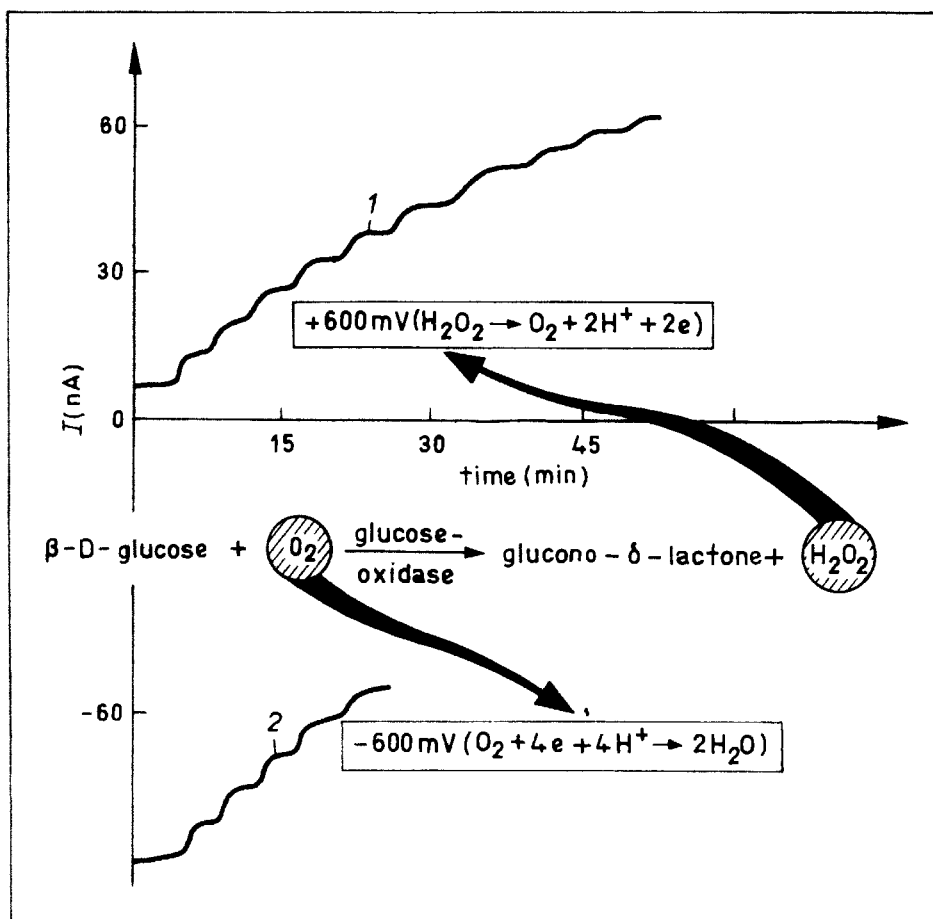


Fig. 41. Response curves of a glucose electrode with indication of hydrogen peroxide formation (1) and oxygen consumption (2) upon stepwise glucose injection.

between the relatively large O_2 base current and the current after O_2 consumption is evaluated (Fig. 41) (e.g., Tran-Minh and Broun, 1975). The sensitivity is thus limited. Furthermore, this method is not suited for solutions with varying oxygen content, since the oxygen probe detects the sum of the oxygen consumption in the enzyme membrane and the change in the bulk solution. This is especially important for blood glucose determination, where deoxyhemoglobin binds a part of the oxygen in the solution and thus simulates a higher glucose concentration. Therefore some companies selling glucose analyzers, e.g. Beckman (USA), prescribe the use of plasma instead of whole blood. This requires centrifugation prior to measurement. To obtain true values with venous

blood, a difference measurement with an enzyme-free O_2 sensor is necessary (Updike and Hicks, 1967).

To compensate for the O_2 consumption in the measuring cell during the measurement of blood samples, Reitnauer (1977) continuously perfused the solution with air. In this case the reproducibility may be affected by partial O_2 oversaturation.

Romette et al. (1979) attempted to solve the problem of varying oxygen tension by using an enzyme membrane with high oxygen solubility. The membrane is prepared by spraying a mixture of GOD, gelatin, and glutaraldehyde onto a hydrophobic polypropylene membrane of 6 μm thickness pretreated with 0.5% lauroyl sulphate. The membrane is saturated with air prior to each measurement to ensure that only oxygen from inside the membrane is consumed in the concentration range 0–22 mmol/l glucose. However, the oxygen concentration in the membrane (20 times higher than in water at the same partial pressure) is not sufficient for *in situ* measurement in fermenters (Romette, 1987).

By measuring current-potential curves, Clark and Duggan (1982) avoided the high stationary state oxygen consumption. In this way it was possible to cover a glucose concentration range of 0–55 mmol/l at a dilution of 1:15.

(ii) *Limitation of the Dynamic Range by Oxygen.* Air-saturated aqueous solutions contain about 7 mg/l oxygen. This is sufficient to oxidize 79 mg/l glucose in the GOD layer. Owing to the more rapid diffusion of oxygen into the membrane system as compared with glucose, in air-saturated solution up to 3 g/l glucose may be detected. Application of an additional diffusion barrier of polyurethane in front of the GOD layer expands the linear measuring range up to 20 g/l (Scheller and Pfeiffer, 1978) (Fig. 42). This approach appears to be particularly useful for implantable glucose sensors.

In order to overcome O_2 limitation, Abel et al. (1984) modified the sensor geometry by covering the usual cuprophane dialysis membrane of the sensor by a hydrophilic (polyurethane, cellulose acetate) and a perforated hydrophobic (polyethylene) membrane (Fig. 43). The diffusion of glucose is limited to the perforated region while oxygen, by nonlinear diffusion, reaches the enzyme membrane via a substantially larger surface area. This approach has been expanded by the concept of the 'two-dimensional' enzyme electrode (Gough et al., 1985). Whereas glucose may diffuse only axially into the enzyme membrane, oxygen is allowed to diffuse axially as well as radially (Fig. 44). For this purpose

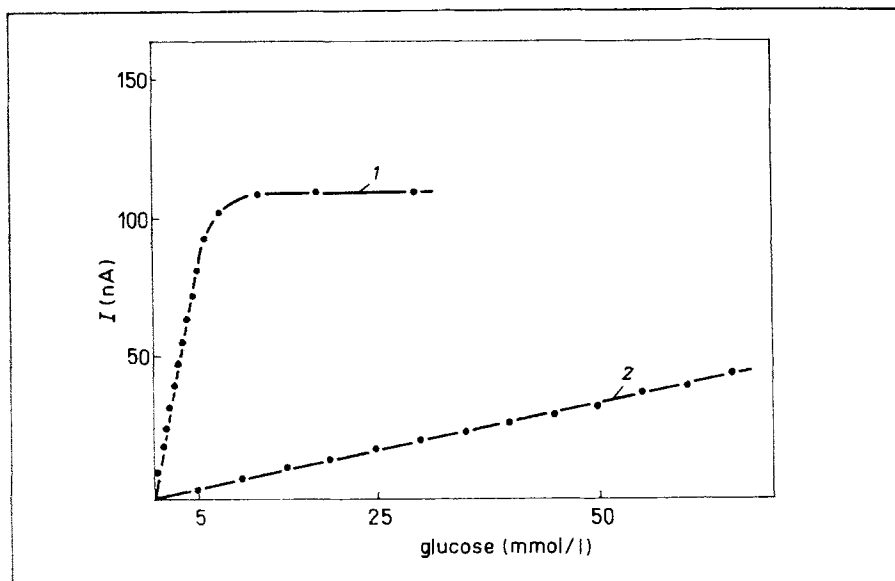


Fig. 42. Comparison of the concentration dependences of the stationary currents of glucose electrodes using gelatin membrane-entrapped GOD sandwiched between two dialysis membranes (1) and with an additional polyurethane membrane next to the solution (2).

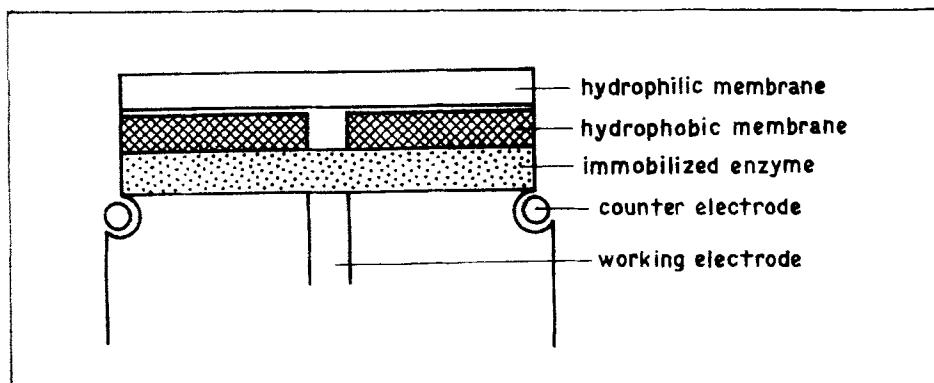


Fig. 43. Enzyme electrode for glucose determination in undiluted biological fluids. (Redrawn from Müller et al., 1986).

the enzyme layer was surrounded by a glucose-impermeable but oxygen-permeable coating.

The dynamic range of *in situ* analysis in fermenters may be restricted by oxygen limitation in the culture broth. To overcome this restriction, Enfors (1981, 1982) developed an oxygen-stabilized glucose electrode

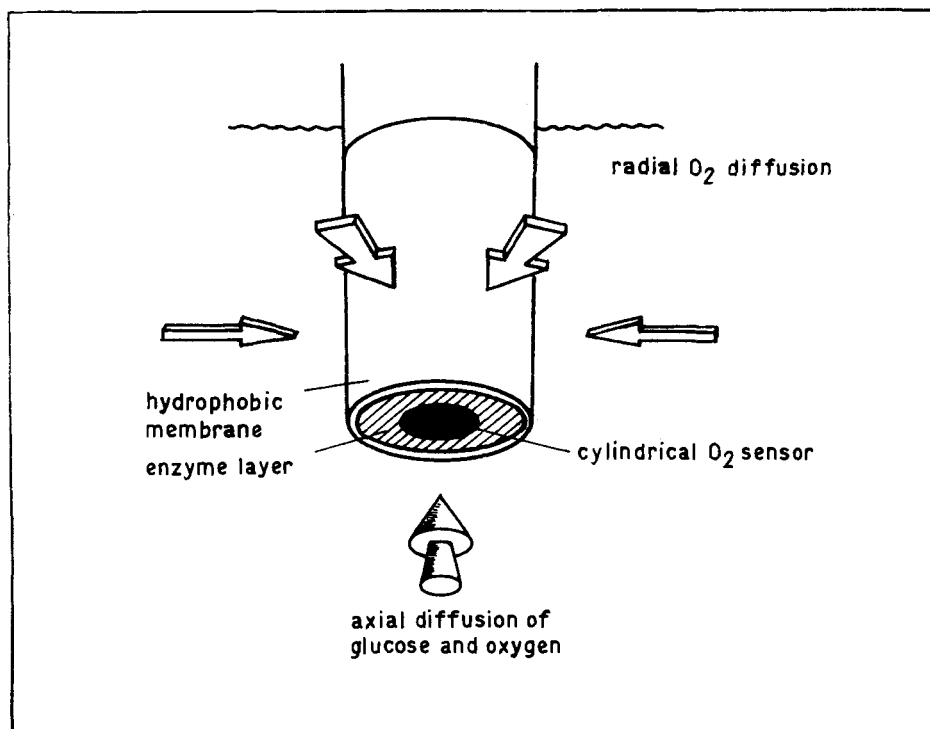


Fig. 44. Schematic layout of a two-dimensional enzyme electrode for glucose. (Redrawn from Gough et al., 1985).

using a galvanic, oxygen-generating electrode placed directly in the enzyme membrane. A common O₂ probe serves as reference electrode. The O₂ consumption caused by glucose oxidation is compensated by electrolytic formation of O₂ from water. The sensor is therefore suitable for solutions with varying oxygen content. Even in anaerobic media the required cosubstrate may be formed electrolytically. The sensor has been successfully employed for continuous *in situ* glucose measurement during *Candida utilis* fermentation (Enfors, 1982).

In an 'externally buffered' enzyme electrode (Fig. 45), substrate-free buffer is continuously pumped between the dialysis membrane and the enzyme layer (Cleland and Enfors, 1984), i.e., the sample is diluted before it reaches the enzyme. The intensity of the buffer flow may be used to adjust the measuring range and sensitivity. The configuration of the sensor permits it to be sterilized. While the membrane is protected by continuously flowing buffer, the rest of the sensor can be sterilized for 1 h in a solution of 95% ethanol and 5% H₂SO₄.

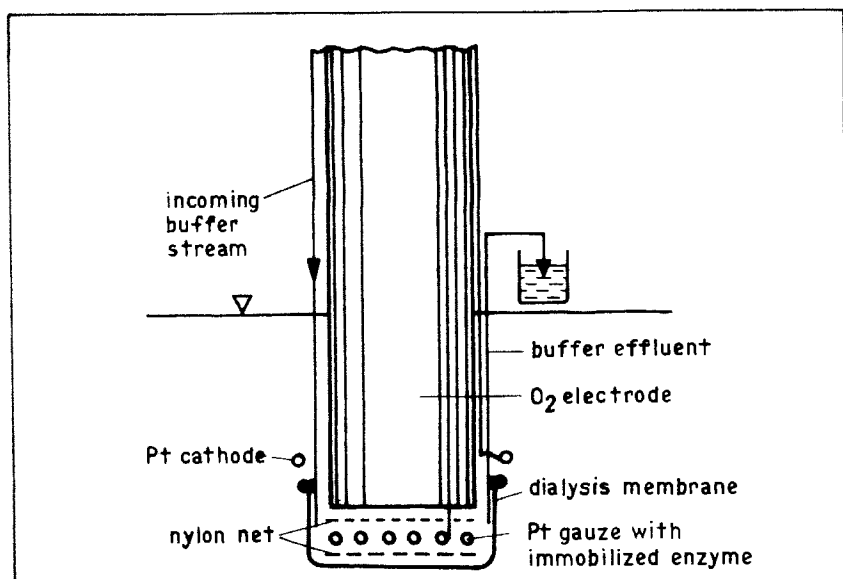


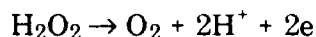
Fig. 45. Externally buffered enzyme electrode. (Redrawn from Cleland and Enfors, 1984).

For routine analysis, flow systems containing enzyme electrodes exhibit various advantages: the setup of the apparatus is simple and may be automated and a high sampling frequency is possible.

Flow systems with oxygen-indicating GOD electrodes have been studied by Macholán et al. (1981) and Pacáková et al. (1984). The enzyme was fixed to polyamide and gelatin, respectively. With aqueous sample solutions a measuring frequency of 30/h and 60/h has been obtained. Biological samples, like blood, serum or plasma, have not been processed.

Glucose sensors based on the O_2 consumption by immobilized microbes will be discussed in Section 3.3.

(iii) *Amperometric Product Indication.* Glucose measurement by means of the anodic oxidation of H_2O_2 according to the process



gives a current signal that rises with increasing concentration (see Fig. 41). The base current is about 50 times lower than for oxygen detection. Accordingly, a considerable sensitivity enhancement can be realized. The lower detection limit is 10 nmol/l hydrogen peroxide.

This principle has been applied by Guilbault and Lubrano (1973) to

GOD entrapped in polyacrylamide. The measuring range was 0.5–10 mmol/l. For sensor preparation the electrode bearing a nylon net of 90 μm thickness was covered with a thin film of enzyme gel (1800 U GOD in 1 ml gel solution consisting of 1.15 g N,N'-methylene bisacrylamide, 6.06 g acrylamide and 5.5 mg riboflavin in 50 ml water) and irradiated with a 150 W actinic electric light bulb for 1 h. Before and during polymerization, oxygen was removed by nitrogen purging.

Interestingly, the authors obtained reproducible results by simply dipping the sensor into the unstirred sample solution.

Mascini and Palleschi (1983a) obtained a stable glucose sensor by using GOD immobilized on nylon. The nylon netting had a thickness of 100 μm and a free surface fraction of 35%. Before binding of lysine as a spacer, the nylon was activated in dry methanol containing dimethylsulfate. GOD was bound after treatment with 12.5% glutaraldehyde for 45 min. This membrane was stable for more than 6 months.

The functional stability of GOD membranes has also been enhanced by coupling with an asymmetric ultrafiltration membrane (Koyama et al., 1980). The GOD-cellulose acetate membrane used was prepared as follows: 250 mg cellulose triacetate was dissolved in 5 ml dichloromethane, the solution was mixed with 0.2 ml 50% glutaraldehyde and 1 ml 1,8-diamino-4-amino methyl octane and sprayed onto a glass plate. After three days the membrane was removed from the support and immersed in 1% glutaraldehyde solution for 1 h at 35°C, rinsed with water and exposed for 2–3 h to phosphate buffer, pH 7.7, containing 1 mg/ml GOD. The membrane was then treated with sodium tetraborate, rinsed with water and stored at 4–10°C until use. It was combined with the ultrafiltration membrane in the following way: 20 mg cellulose diacetate was dissolved in 35 g formamide and 45 g acetone and cast on a glass plate. At room temperature the solvents evaporated within a few seconds and a membrane of about 30 μm thickness remained, which was kept in ice water for 1 h before application in the sensor.

The sensor using these combined membranes was stable for more than 100 days (at 0.196 mmol/l glucose) whereas without the ultrafiltration membrane only 15 days' stability could be achieved. The membrane caused only a minor delay in sensor response.

Several reducing substances that are present in biological media and are anodically cooxidizable with H_2O_2 may contribute substantially to the current signal. Most prominent are ascorbic acid, uric acid, paracetamol, and glutathione. In dependence on medication and metabolism the concentration of these compounds may vary within a wide range.

Various efforts have been made to eliminate these electrochemical disturbances. Lobel and Rishpon (1981) employed a negatively charged ion exchange membrane to suppress the permeation of ascorbic acid and uric acid. The membrane (molecular cutoff 1000 or 6000–8000) was modified by immersing it in an aqueous solution of 10% triazenyl (brilliant orange) dye and 3% Na_2CO_3 at pH 10.5 and room temperature for 2 h. It was able to eliminate disturbing signals up to 0.0852 mmol/l ascorbic acid and 0.464 mmol/l uric acid. The influence of glutathione and bilirubin was not diminished.

Harrison et al. (1988) have shown that GOD-modified platinum electrodes can be effectively protected from electrode fouling by dip-coating with a perfluorosulfonic acid polymer. The dip-coating formed a polymer layer of 2 μm thickness on the electrode. After 6 days of continuous *in vitro* measurement in whole blood at 37°C the sensitivity was decreased by only 6%. Interferences from electroactive anions were reduced due to the Donnan exclusion of these species. Nevertheless, differential measurements were necessary for complete elimination of interferents.

To perform highly sensitive glucose measurements in biological samples, Thevenot et al. (1979) evaluated the current difference between an enzyme sensor and one with no enzyme. They used a collagen membrane from Centre Technique du Ciur (Lyon, France), which was activated by the acyl azide procedure and loaded with GOD (Fig. 46). The permeability of the thick collagen membrane was only slightly changed by this immobilization, so that both electrodes exhibited the same sensitivity for electrochemically interfering substances. The lower detection limit of the difference arrangement was 0.01 mmol/l glucose, the linear range being 0.1 $\mu\text{mol/l}$ –2 mmol/l. The setup is being applied in the microprocessor-based 'Glucoprocasseur' analyzer (Solea-Tacussel, Lyon). This device responds to a glucose sample within 30 s and is principally intended for use in food analysis.

Assolant-Vinet and Coulet (1986) used a preactivated 'Biodyne Immunoaffinity Membrane' (Pall, USA) to cover two electrodes working in differential mode. Immobilization was performed by simply dropping the enzyme solution onto the membrane, i.e., the biosensor-users may prepare the enzyme membranes themselves. Good results were obtained with GOD, lactate oxidase, and oxalate oxidase (Coulet, 1987).

In order to exclude disturbing substances, Newman (1976), Tsuchida and Yoda (1981), and Palleschi et al. (1986) covered the platinum electrode by a H_2O_2 selective asymmetric cellulose acetate membrane. The membrane (thickness, 15.3 μm) was prepared from acetyl cellulose

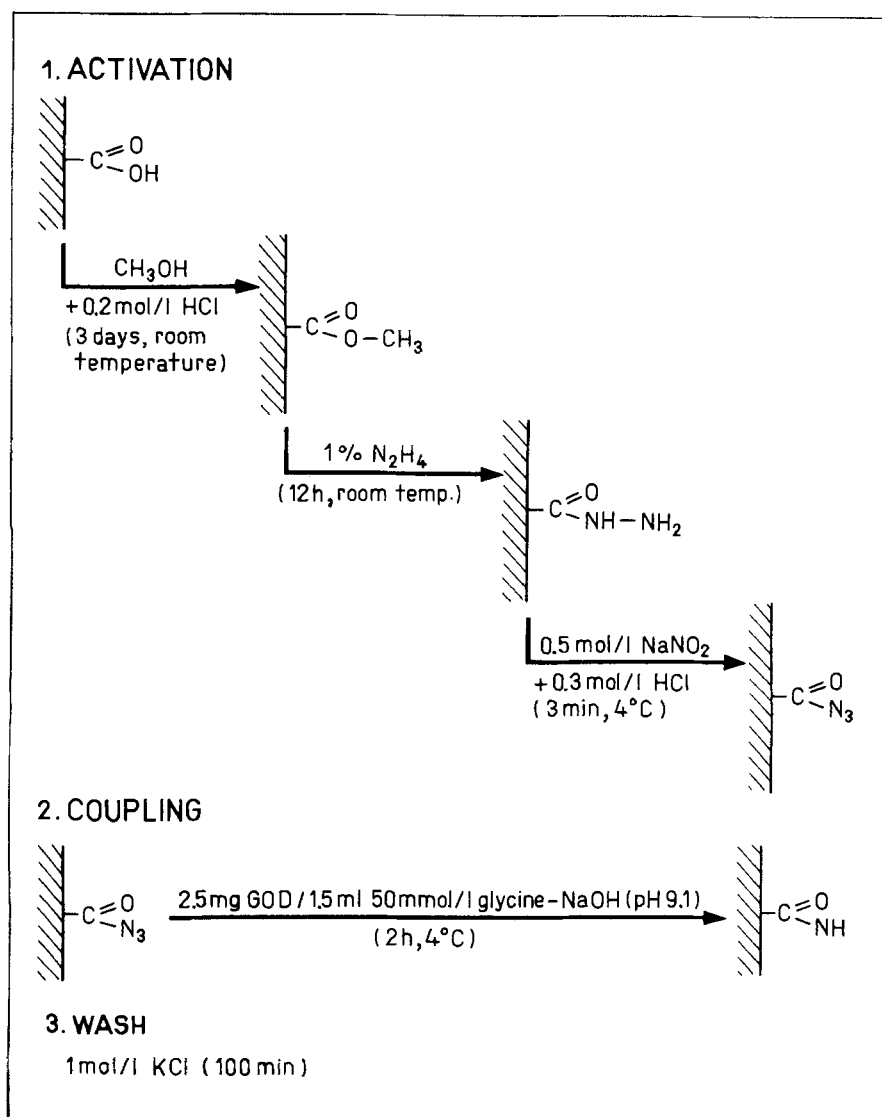


Fig. 46. Reaction scheme for the preparation of GOD-collagen membranes.

dissolved in acetone/cyclohexanone. A mixture of 400 U GOD, 2 mg BSA, and 0.25% glutaraldehyde in 0.15 ml 0.05 mol/l acetate buffer, pH 5.1, was dropped onto the porous side of the asymmetric membrane. After 24 h the membrane was rinsed with glycine buffer (Tsuchida and Yoda, 1981). Most interfering substances can be prevented from reaching the electrode by using this mechanically rather unstable membrane. It is being applied in the glucose GLUCO 20 analyzer of the Japanese Fuji

Electric company (Osawa et al., 1981). The membrane developed by Newman (1976) is being used in the 23 A glucose analyzer (Yellow Springs Instrument Co., USA, see Section 5.2.3). Lindh et al. (1982) showed that the cellulose acetate membrane is not truly selective for H_2O_2 , but only decreases the permeation of larger molecules. The sensor was 3 times more sensitive to the glycolysis inhibitor paracetamol than to glucose. The membrane permeability was shown to depend on membrane age.

Palleschi et al. (1986) have shown that an electrode covered with a cellulose acetate membrane with a molecular cutoff of 100 is not entirely selective for H_2O_2 but exhibits a pronounced response to paracetamol. On the other hand, Petersson (1988a) reported that no effect is found from ascorbic acid, glutathione, and paracetamol when the 'high-rejection' Yellow Springs GOD membrane is applied in an FIA device with reduced frequency. Furthermore, at electrode potentials below +300 mV the paracetamol signal is completely suppressed whilst the sensitivity for hydrogen peroxide remains half as high as that at +600 mV.

By combination of the derivative (rate) measuring regime with an additional diffusion barrier on the back side of the enzyme membrane, Scheller and Pfeiffer (1978) achieved accurate and rapid glucose determination in whole blood and serum even without using a permselective membrane. In contrast to stationary measurement, in the rate method the maximum slope of the current-time curve is evaluated. In addition to the advantage of a shorter response time (3 s) this procedure excludes virtually all oxidizable substances. As is shown by comparison of I-t curves of pure glucose solution and physiological solution (Fig. 47) the disturbing substances diffuse through the membrane more slowly than H_2O_2 .

The GOD membranes were prepared as follows: a 5% gelatin solution was incubated for 1 h at room temperature and then stirred for 1 h at 40°C. 1 mg GOD (*P. notatum*, 46 U/mg) was mixed with 40 μ l of the solution and the resulting solution was cast on 1 cm² of a planar support. After drying for 6 h the membrane was removed and stored under dry conditions at 4°C until use.

One GOD-gelatin membrane can be used for more than 2000 measurements. These membranes have been employed in the 'Glukometer GKM' glucose analyzer (ZWG Berlin, GDR).

Wollenberger et al. (1986) proposed the elimination of interfering substances by using laccase in a glucose sensor. The electrode involved a layer of coimmobilized GOD and laccase in a gelatin membrane.

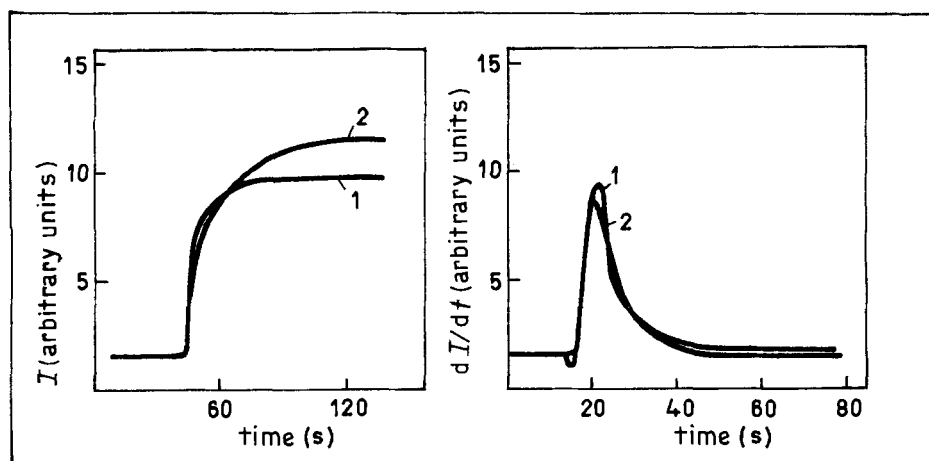


Fig. 47. Comparison of stationary and kinetic response curves of the measurement of glucose standard solution and serum. 1: standard solution, 5.5 mmol/l; 2: control serum Serulat (VEB Sächsisches Serumwerk Dresden, GDR), 4.8 mmol/l.

Hexacyanoferrate(III) was added to the samples in order to oxidize reductive interferences such as ascorbic acid, and the hexacyanoferrate(II) formed was reoxidized by a laccase-catalyzed reaction. This approach appears to be particularly useful for samples with extreme concentrations of oxidizable compounds, such as urine.

The sensitivity of glucose determination by means of hydrogen peroxide detection may be decreased by the catalase that contaminates several GOD preparations. If no catalase-free GOD is available, this problem can be largely overcome by adding catalase inhibitors such as sodium azide or aminotriazole to the background solution.

A glucose sensor based on the detection of hydrogen peroxide vapor has been developed by Kessler et al. (1984). A gold anode was covered by a lipophilic membrane and GOD (Fig. 48). Minute amounts of gaseous H_2O_2 diffuse through the membrane to the electrode. The membrane contains ion carriers to remove protons liberated during the anodic oxidation of H_2O_2 . Currents as low as $1\text{--}20\text{ pA/cm}^2$ have been measured: i.e. the electrical resistance of the sensor approximates that of a potentiometric electrode. The system had a drift of less than 1 mV/24 h , an operational stability of 3 months, and a low permeability for glucose, providing an extremely low oxygen demand. These features make the sensor promising for use in *in vivo* application or implantation.

In spite of the problem of interferences, the direct electrochemical

oxidation of H_2O_2 has so far been the most successful method for glucose determination using biosensors. The first commercial glucose analyzer based on an enzyme electrode was introduced in 1975 by Yellow Springs Instrument Co. It is based on electrochemical H_2O_2 indication. The same principle is used in the on-line analyzer described by Fogt et al. (1978), which has been integrated in a computer-controlled feedback system to form the 'Biostator GC IIS' (Life Science Instruments, USA).

The highest sample frequency achieved up to now has been obtained in a computer-controlled flow injection analysis (FIA) system (Olsson et al., 1986b) using an amperometric flow-through cell (Bertermann et al., 1981). A Pt electrode of 0.5 mm diameter (VEB Metra Radebeul, GDR) has been used as hydrogen peroxide detector. GOD was immobilized in polyurethane (thickness, 0.02 mm, enzyme loading, 50 U/cm^2) and

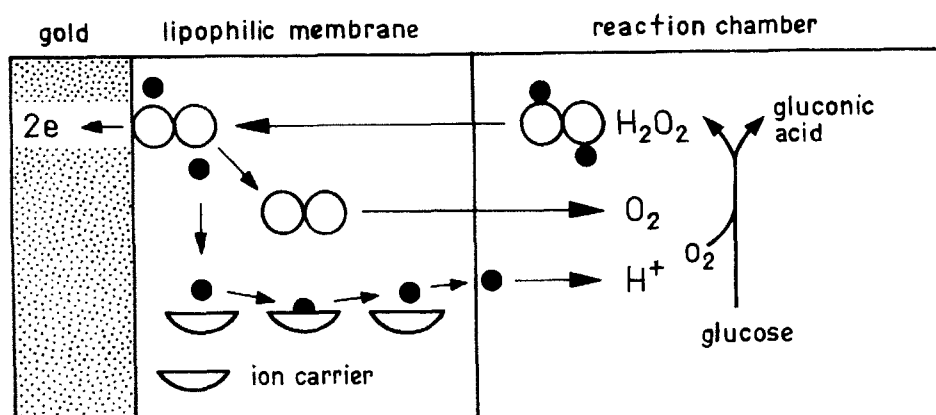
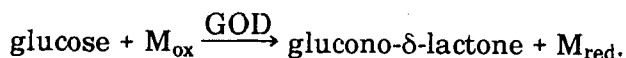


Fig. 48. Schematic view of a glucose electrode based on sensing of gaseous hydrogen peroxide. (Redrawn from Kessler et al., 1984).

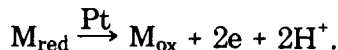
sandwiched between two dialysis membranes (Nentwig et al., 1986). The high enzyme activity in the membrane provides diffusional control of the sensor response (Scheller et al., 1983a). Optimal sample frequency and sensitivity were calculated by using a mathematical model for diffusion-limited amperometric enzyme electrodes in FIA. The experimentally obtained measuring range of 0.01–100 mmol/l and sensitivity of $0.5 \mu\text{A l/mol}$ were in good agreement with the theory. At an injection volume of $1.5 \mu\text{l}$ the sample frequency was 300/h (Fig. 49); the coefficient of variation was 0.5% ($n = 40$).

As an alternative to the use of the natural cosubstrate, oxygen, the

employment of artificial electron acceptors in glucose sensors has been intensively studied:



The reduced electron acceptor, M_{red} , is reoxidized at the electrode:



The mediator 2,6-dichlorophenol indophenol (DCPIP) has been used by Mindt et al. (1973). Mor and Guarnaccia (1977) employed the $[\text{Fe}(\text{CN})_6]^{3-}/[\text{Fe}(\text{CN})_6]^{4-}$ couple and performed difference measurements with a GOD sensor and an auxiliary electrode to eliminate the influence of mediator-consuming side reactions. The concentration of hydroquinone formed from benzoquinone in the GOD reaction has been measured by Geppert and Asperger (1987).

Mullen et al. (1985) circumvented the problem of oxygen demand by using quinoprotein glucose dehydrogenase from *Pseudomonas* or *Acine-*

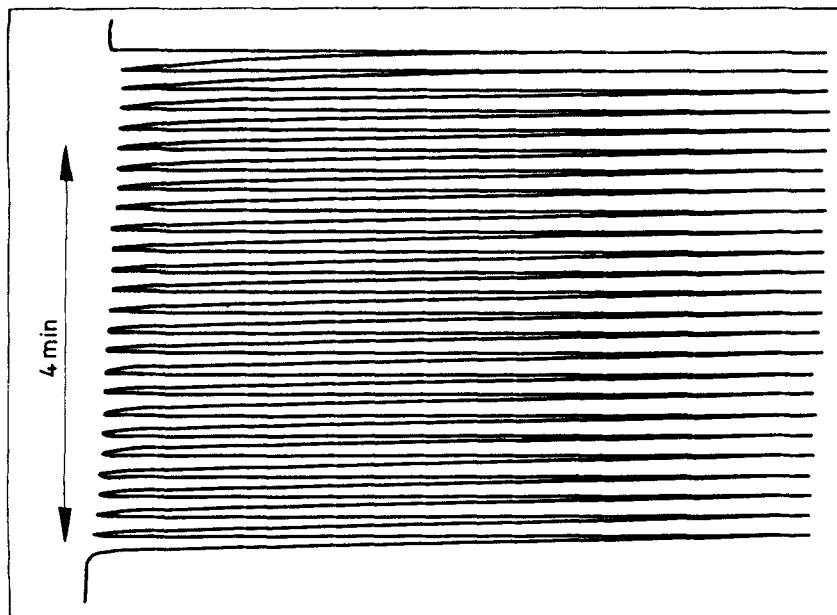


Fig. 49. Response of an FIA manifold provided with a glucose electrode containing polyurethane-immobilized GOD to injection of 25 glucose samples of 1 mmol/l. The measuring frequency was 200/h. (Redrawn from Olsson et al., 1986b).

tobacter species in combination with DCPIP and phenazine ethosulfate (PES). The latter was unstable above 1.2 mmol/l glucose but provided a substantially lower response time (3–5 min) than DCPIP (30–60 min). In aerobic solution disturbances were caused by autoxidation of the reduced mediators.

Recently, Kotowski et al. (1988) immobilized GOD by glutaraldehyde in a conductive lecithin polypyrrole bilayer membrane (BLM). The membrane was formed by polymerization of pyrrole in the presence of ferric chloride and was arranged between two electrodes. Addition of glucose leads to the formation of anodic peaks at 200 mV which reflect the electron transfer from reduced GOD to polypyrrole in the BLM system. The peak current exhibits a nonlinear dependence on the concentration of glucose between 1 and 20 mmol/l.

3. Thermistors and Optoelectronic Sensors

Tran-Minh and Vallin (1978) covered a thermistor directly with a membrane containing GOD and catalase in a mixture of glutaraldehyde and BSA. A reference thermistor covered by a BSA membrane served to eliminate temperature variations. The response time of the device (10 s) resembles that of enzyme electrodes; the time to reach the baseline was not reported. The authors adjusted a defined oxygen concentration by adding H_2O_2 to the measuring solution. By the action of catalase an oxygen partial pressure was created that was independent of temperature and ambient air pressure. Depending on the H_2O_2 concentration, the linear measuring range could be shifted by almost one order of magnitude. The thermistor was stable for 2–3 weeks.

Muehlbauer et al. (1989) attached a Bi/Sb thermopile directly to a membrane containing immobilized GOD and catalase. The basic sensor was assembled by vacuum deposition of the metals. The dynamic behavior of the thermoelectric glucose sensor was modeled. The results properly reflected the sensor response.

Hydrogen peroxide formed in the GOD reaction can also be detected by chemiluminescence (Seitz et al., 1982). Although this method is very sensitive (detection limit 7 nmol/l glucose) it has not been used practically because expensive materials and pretreatment of physiological samples are necessary.

Lowe et al. (1983) utilized the detection of protons produced during glucose oxidation by an optoelectronic sensor. Glucose oxidase was coupled to a transparent cellophane membrane together with the pH sensitive triphenylmethane dye bromocresol green. The membrane was

fixed in a flow-through cell between a light emitting diode (LED) and a photodiode (see Fig. 8). The change of the proton concentration during enzymatic glucose oxidation changes the color of the dye from blue-green to yellow. This leads to a change of the output voltage of the photodiode, which was linearly dependent on glucose concentration in the range 0–70 mmol/l. One concentration decade corresponds to a voltage change of 1.5 V.

In an analogous approach, GOD immobilized by crosslinking with glutaraldehyde and BSA has been applied to a fluorescence optode for O_2 , covered with a cellophane membrane (Opitz and Lübbers, 1987). The relative fluorescence intensity as photometrically measured was proportional to glucose concentration up to 7 mmol/l.

Recently, GOD was immobilized on the tip of a fluorescence pH sensitive optode (Trettnak et al., 1989). This sensor responded to 0.1–2 mmol/l glucose within 10 min.

3.1.1.3 Enzyme-Chemically Modified Electrodes

The direct fixation of the biocatalyst to the sensitive surface of the transducer permits the omission of the ‘inactive’ semipermeable membranes. However, the advantages of the membrane technology are also lost, such as the specificity of permselective layers and the possibility of affecting the dynamic range by variation of the diffusion resistance. Furthermore, the membrane technology has proved to be useful for reloading reusable sensors with enzyme. In contrast, direct enzyme fixation is mainly suited to disposable sensors. This is especially valid for carbon-based electrodes, metal thin layer electrodes printed on ceramic supports, and mass-produced optoelectronic sensors. Field effect transistors may also be envisaged as basic elements of disposable biosensors.

The simplest method of electrode modification is surface adsorption of the enzyme. As early as 1976, Silver reported a glucose microelectrode prepared by adsorption of enzyme to a platinum probe. The initial activity of this sensor was sufficient but it dropped strongly within a few hours.

The presence of various functional surface groups and the high conductivity and porosity of carbon material permit effective enzyme adsorption. Glucose oxidase has been irreversibly adsorbed to a graphite electrode by drying a concentrated enzyme solution on the surface (Ikeda et al., 1984). In the presence of p-benzoquinone an electrocatalytic current was observed at 500 mV vs SCE. The measuring signal was

independent of stirring speed. Analysis of the concentration dependence revealed an agreement between the kinetics of the immobilized and the soluble enzyme. For both forms an activation energy of 45 kJ/mol and a pH optimum of 6.5 were found. The maximum turnover number and the K_M for glucose were also in agreement. The sensor was covered by a thin collodion membrane to increase its operational stability.

Gorton et al. (1985) employed GOD adsorbed on spectral carbon for glucose determination in a wall jet flow-through cell of an FIA system. Up to 120 samples per hour have been measured with good precision. The enzyme electrode was stable for 3–7 days. Coating of the spectral carbon by a Pd/Au layer of 20 μm thickness permits H_2O_2 to be anodically indicated at potentials as low as +350 mV vs SCE. The enzyme activity is not affected by this modification.

In general the functional stability of adsorbed enzymes is relatively poor due to more or less strong desorption. The desorption process may be slowed down by intermolecular crosslinking of the adsorbed enzymes or by covering them with a thin semipermeable membrane.

Ikariyama et al. (1987) constructed a glucose sensor by adsorption of GOD during the electrolytic deposition of platinum black on a microelectrode. The sensor was highly sensitive and had a response time of only 3 s for the stationary signal.

Alternatively, GOD was crosslinked together with BSA by glutaraldehyde on a platinized electrode after sequential adsorption of the proteins (Ikariyama et al., 1988). The peak height after application of a potential pulse of 600 mV was proportional to the concentration of glucose up to 20 mmol/l. The blank signal for glucose-free buffer was as high as that for undiluted blood. Subtraction of the signals resulted in a diminished precision. An advantage of the measuring principle is that it does not require agitation or dilution of the sample and is independent of the sample volume above 2 μl .

GOD has been covalently bound to carbon by glutaraldehyde-coupling to aliphatic amines in graphite paste electrodes (Shu and Wilson, 1976), by carbodiimide-coupling to carboxyl groups (Wieck et al., 1984; Bourdillon et al., 1980), and coupling by means of cyanuric chloride to hydroxyl groups (Ianniello and Yacynych, 1981). The immobilization yield of the first method has been increased by introducing an albumin layer between the electrode surface and the enzyme.

Since the roughness of the carbon surface is unknown, calculation of the surface concentration of enzyme is difficult. In particular, no distinction can be made between mono- and polylayers. A decrease of the

specific activity with increasing enzyme concentration, however, as observed by Razumas et al. (1984), points to the formation of polylayers.

The strong alteration of the microenvironment due to covalent fixation is reflected by a change in the kinetic properties of GOD. With different binding methods the pH optimum may be shifted by up to one pH unit in the acid or alkaline direction as compared with the free enzyme. The apparent K_M value for glucose may vary in the range 3.1–19.1 mmol/l.

Electrodes with covalently bound enzyme usually have a high functional stability. Bourdillon et al. (1982) observed 75% of the initial GOD activity after 30 days of intermittent use of a GOD-carbon sensor. When the enzyme was in continuous contact with glucose, autoinactivation caused an activity decay of 75% within 6 h.

The principle of 'dual electrodes' allowing both amperometric and potentiometric substrate determination has been described by Ianniello et al. (1982a). A reversible redox mediator, such as ferrocyanide, is added to the sample and either the diffusion current at constant potential or the redox potential at zero current is evaluated.

Yacynych et al. (1987) further developed the ECME concept to improve the signal-to-noise ratio and suppress disturbances by electrode-active serum components. Platinum was electrolytically deposited on a part of a porous carbon electrode, thus decreasing the electrode potential for H_2O_2 oxidation to +600 mV as compared with +900 mV for unmodified carbon. GOD was then covalently fixed to the free carbon surface by carbodiimide coupling. Finally, a film that was permeable only to hydrogen peroxide was formed at the electrode by electropolymerization of diaminobenzene. Since the electrode material embodies enzyme, carrier, reactor, and detector on a microscopic scale the authors called this configuration an 'integrated electrochemical biosensor system'. It has been used in an FIA system for glucose determination for up to three months.

For direct fixation of GOD to platinum electrodes, Yao (1983) and Castner and Wingard (1984) treated the sensor surface with aminopropylsilane and crosslinked the enzyme with glutaraldehyde and BSA to the alkylamine groups. The main advantage of this sensor type is its low response time; up to 100 measurements could be performed per hour.

Adsorption or crosslinking of GOD at platinized carbon paper electrodes resulted in a glucose sensor requiring only minute amounts of oxygen (Bennetto et al., 1988). The authors postulated that the electron transfer from the enzyme to the electrode may be affected by adsorbed

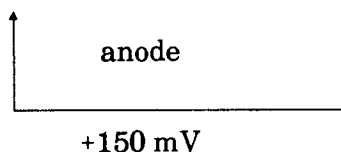
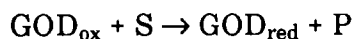
oxygen species. The linear measuring range of the sensor extended up to 25 mmol/l glucose, and a nonlinear response was found between 25 and 80 mmol/l. The time to reach 95% of the current plateau was 1–2 s. At the working potential of 250 mV vs SCE interference by reducing compounds was low.

Simultaneous immobilization of enzymes and mediators renders the measuring procedure completely independent of cosubstrate concentration in the sample and permits a reagentless regime. Except for the analyte all necessary reaction partners are present at the sensor surface. As mentioned in Section 3.1.1.2, Mindt et al. (1973) demonstrated the usefulness of artificial electron acceptors in GOD electrodes. In a patent (Mindt et al., 1971) they suggested introducing the solid mediator in crevices of carbon electrodes and preventing it from leaching out by a dialysis membrane. However, since soluble mediators were used, the electrodes were rapidly exhausted. An alternative approach has been investigated by Ikeda et al. (1985), who integrated benzoquinone (BQ) into a graphite paste electrode by mixing it (3–20%) with graphite powder and paraffin oil. GOD was dropped onto the prepared electrode surface and, after drying, the electrode was covered with a thin collodion membrane. When the sensor was in contact with a measuring solution, equal rates of leaching out and reloading of BQ from the electrode body were reached. Twenty seconds after addition of glucose a hydroquinone oxidation current was observed that was independent of stirring. The sensor was stable for one week with a linear range between 1 and 15 mmol/l glucose ($K_M = 104$ mmol/l). The CV of 5% was rather poor. A principal advantage of the sensor is its independence of the oxygen content of the solution. Matuszewski and Trojanowicz (1988) improved this sensor type by entrapping GOD together with BQ in the carbon paste electrode.

Jönsson and Gorton (1985) adsorbed N-methylphenazinium (NMP^+) onto a spectral carbon electrode containing covalently bound GOD. Whereas the immobilized enzyme was stable for 2 months, fresh NMP^+ had to be adsorbed daily. An important advantage of this sensor is the low required potential, +50 mV vs. SCE, which keeps electrochemical interferences to a minimum.

Cenas and Kulys (1981) described an elegant method for modifying electrode surfaces. A layer of TCNQ was deposited on a glassy carbon electrode by anodic oxidation of Li^+TCNQ^- . Similarly, a layer of Li^+TCNQ^- may be formed by cathodic reduction of TCNQ. An enzyme solution, e.g. GOD, was entrapped on this mediator layer by a dialysis

membrane. A defined distance between the electrode and the membrane was provided by a nylon net. Both TCNQ and TCNQ^- are effective electron acceptors for reduced GOD ($k_{\text{ox}} \approx 10^4 \text{ l/mol}\cdot\text{s}$). The limiting current region of the electrochemical oxidation of the reduced forms, i.e. TCNQ^- and TCNQ^{2-} , occurs at +500 mV and +150 mV vs SCE, respectively. Therefore, addition of glucose causes an anodic current according to:



The sensor responded linearly to glucose in the range 0.5–5 mmol/l. In air-saturated solution the current was 62% lower than under anaerobic conditions. The maximum current at glucose saturation was linearly dependent on the concentration of GOD in the measuring chamber of the electrode, i.e., the response was kinetically controlled. The apparent K_M for glucose increased from 0.48 to 4 mmol/l with increasing GOD concentration, indicating the influence of diffusion. When the electrode was disconnected, the charge transferred by the enzyme to the mediator accumulated. A high current peak was obtained when the enzyme electrode was connected again. This 'discharge' was completed within a few minutes. Evaluation of the peak current increased the sensitivity of the measurement by a factor of 10.

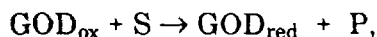
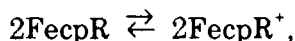
Adsorption of the redox polymers polyhydroxyquinone or 1,4-benzoquinone polyvinylpyridinium bromide from ethanolic solution to glassy carbon also resulted in catalysis of the electron transfer from GOD to the electrode (Kulys et al., 1982). Interestingly, the same effect was observed using mixtures of the enzyme with carbon black. This is obviously due to the presence of quinoidic groups at the surface of the carbon particles.

A very successful mediator-chemically modified electrode (MCME) has been developed by using ferrocene derivatives (Cass et al., 1984). Ferrocene (bis-cyclopentadienyl iron) and its derivatives (FecpR) combine the good electrochemical behaviour of ferrocyanide with the possi-

bility of structural modification of organic dyes and the effective electron acceptance from GOD.

For preparation of the GOD electrode a highly porous graphite foil of 1 mm thickness (Union Carbide, USA) is oxidized in air at 100°C. A 0.1 mol/l 1,1'-dimethylferrocene solution in toluene is dropped onto the electrode and the solvent allowed to evaporate. GOD is covalently bound to the oxidized carbon surface by carbodiimide coupling and the surface is covered by a polycarbonate membrane of 0.03 μm thickness. Before use, the electrode is conditioned in 7 mmol/l glucose solution for 10 h with an applied potential of +160 mV.

Reduced dimethylferrocene is nearly insoluble in water and has a half wave potential of 100 mV vs SCE, which diminishes electrochemical interferences in glucose determination. The adsorbed mediator becomes active as an electron acceptor only by anodic oxidation at +160 mV to the ferricinium ion (FecpR^+):



The main advantage of working with this mediator in a glucose sensor is the higher K_M value for glucose as compared with that when O_2 acts as cosubstrate. In the study cited above this led to a linear measuring range of 1–30 mmol/l glucose. Above 30 mmol/l the current increased nonlinearly, reaching a saturation value at ca. 70 mmol/l. In a stirred solution the stationary current increased by 10% in comparison with an unstirred one. The time to reach 95% of the stationary current was 60–90 s. Since GOD reoxidation is considerably more rapid with ferricinium than by oxygen, the competition by air oxygen was of minor influence (about 4%). The glucose signal was independent of pH because no protons take part in the electrode reaction. The sensor has been used for glucose measurement in undiluted blood and plasma, food samples, and fermentation broth (see Sections 5.2 and 5.4).

The glucose sensor based on ferrocene-modified carbon has been perfected at Cranfield Institute of Technology (UK) and is being mass-produced by Genetics International (UK) as a disposable blood glucose sensor. Carbon paste containing carbon powder, ferrocene, an organic binder, and possibly the enzyme is fixed to a PVC support by screen printing. In analogy to the enzyme electrode, an Ag/AgCl reference and

a counter electrode are also printed on the support. The sensor is used as disposable material in the ExacTech glucose test kit analyzer (Higgins et al., 1987). The technology also permits production of microstructures of multiple sensors applicable for the determination of concentration profiles or for statistical evaluation of multiple measurements (Fig. 50).

Turner et al. (1987b) proposed to substitute tetrathiafulvalene for ferrocene in a glucose sensor. GOD was covalently bound to a mediator-loaded pyrolytic carbon electrode by using carbodiimide. The sensor exhibited a linear calibration graph for glucose up to 25 mmol/l with a typical time to reach 90% of steady state response of 3–5 min. At 80 mmol/l the response decreased by 50% within 1.5 h.

D'Costa et al. (1986) bound quinoprotein glucose dehydrogenase via carbodiimide to a graphite foil impregnated with ferrocene monocarboxylic acid. This combination is especially useful since the electron transfer is extremely fast ($k_{\text{ox}} = 9.3 \cdot 10^6 \text{ l/mol}\cdot\text{s}$). Although only 0.5% enzyme activity as compared with the GOD sensor has been used the high reaction rates of this system resulted in a higher sensitivity. The response time for stationary current measurement was only 10–20 s and 80% of the initial activity were still present after 13 h at 30°C. The measuring value was independent of pH and O_2 concentration. The linear range was 0.5–4.0 mmol/l.

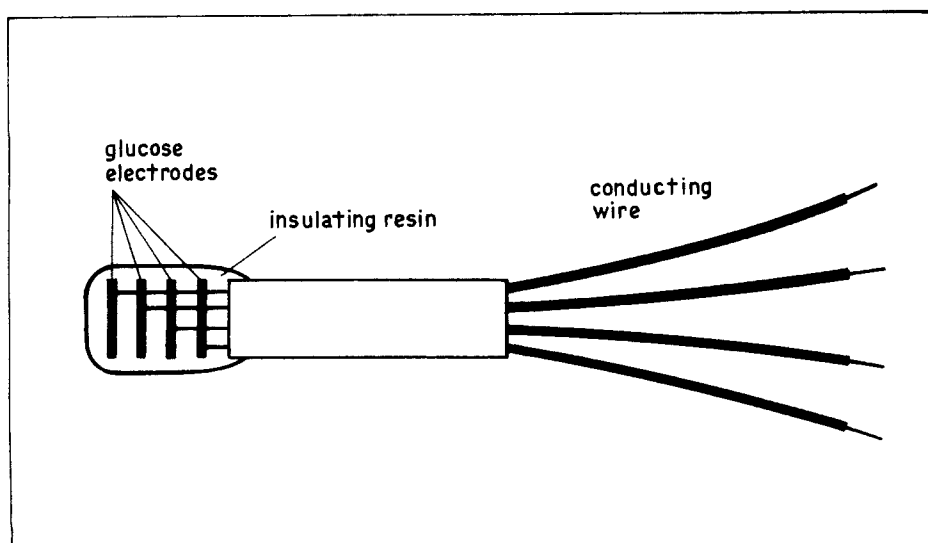


Fig. 50. Layout of the sensor from Cranfield Institute of Technology (U.K.) for the measurement of glucose concentration profiles in meat.

An ordered arrangement of GOD and ferrocene on a platinum electrode has been obtained by using the Langmuir-Blodgett (LB) technique (Moriizumi et al., 1988). Monomolecular films on electrodes were made by mixing the mediator with lipid followed by adsorption of GOD on the film at the trough surface. The quantity of GOD could be flexibly controlled by the number of LB films, thus giving sensors of different sensitivity.

The entrapment of enzymes within electropolymerized films offers the possibility of attaching the biocatalysts to conducting surfaces. Polypyrrole and polyaniline have been used as semiconducting electrode coatings. Electropolymerization of pyrrole proceeds via a highly reactive π -radical cation which reacts with other pyrrole molecules to form chains with mainly α, α' -coupling. Since the polymer is positively charged, anions from the solution are accumulated in the film. Foulds and Lowe (1986) conducted the electropolymerization at a platinum electrode at +800 mV in deaerated aqueous solution containing 0.2 mol/l pyrrole and 0.13 μ mol/l GOD. After about 2.5 h a film containing up to 125 mU/cm² GOD was formed. The negative gross charge of GOD at pH 7 decisively influenced the efficiency of enzyme entrapment in the positively charged matrix. Using H₂O₂ indication, this sensor exhibited a nonlinear calibration curve for glucose up to 100 mmol/l in air-saturated solution. The response time was 20–40 s and the useful lifetime 20 days.

An analogous glucose sensor has been developed by Umana and Waller (1986). Glucose measurement was carried out by reduction of iodine formed from iodide in a molybdate-catalyzed reaction. The current did not reach a stationary value but increased linearly with time. Obviously the main part of glucose oxidation was catalyzed by GOD leached out into the measuring solution. Therefore the sensitivity dropped to 25% of the initial value after 5 measurements and after 10 days the enzyme was completely exhausted.

Redox polymers for enzyme entrapment have been developed in order to combine the advantages of electropolymerization technology with those of MCME. The group led by Lowe in Cambridge (UK) (Hall et al., 1986) employed ferrocene derivatives of pyrrole for immobilization by electropolymerization. In these systems ferrocene takes the electrons up from the reduced prosthetic group of the enzyme and conducts them to the electrode. Shaojun et al. (1985) studied the direct polymerization of ferrocene derivatives; the redox polymer formed might be suitable for coupling with enzymes.

Based on investigations of enzyme coupling to semiconducting gels

carried out in Berezin's group (Varfolomeev et al., 1980), Kulys and Svirnickas (1980a) pioneered the coupling of conducting polymers with oxidoreductases for analytical purposes. These authors prepared organic metal electrodes by pressing a powder of the complexes of NMP^+ or N-methylacridinium (NMA^+) with the anion radical TCNQ^- . GOD was entrapped in a nylon net at the electrode surface by a semipermeable membrane. After addition of glucose to the measuring solution a current in the TCNQ^{2-} oxidation region (50–400 mV vs Ag/AgCl) was observed. The current was proportional to glucose concentration up to 2.6 mmol/l and did not depend on pH between pH 5.1 and 7.8. When an organic metal electrode with adsorbed GOD was used, the stationary current was only 1/8 of that found with GOD (18 $\mu\text{mol/l}$) in the solution surrounding the electrode. The maximum current at glucose saturation was linearly dependent on GOD loading. This finding, together with the charge accumulation observed with the TCNQ^- -modified electrode (see above), indicates that the charge transfer between GOD and the electrode was due to dispersed mediator particles. This hypothesis was verified by calculation taking account of the low solubility of TCNQ , 69 $\mu\text{mol/l}$, and the high charge transfer rate constant.

Based on the investigations by Kulys et al., three types of TCNQ electrodes using different cation radicals have been developed (Albery et al., 1985). Paste electrodes contain a plastic slurry formed by evaporation of the solvent from a mixture of organometallic compound and PVC in tetrahydrofuran. Evaporation is conducted in the cavity of a disk electrode or on the surface of a glassy carbon electrode. Furthermore, the surface of a crystal-like TCNQ needle has been applied in a rotating disk electrode arrangement. The enzyme was entrapped by covering with a dialysis membrane. The best results have been obtained with electrodes formed from TCNQ^- and NMP^+ or tetrathiafulvalene (TTF). Owing to the extremely low background current the measuring range was as large as 50 $\mu\text{mol/l}$ –10 mmol/l glucose. After 30 days of continuous use, the sensitivity dropped by only 20%, the decrease being due to enhanced diffusion resistance rather than to enzyme inactivation.

By analyzing the dynamic behaviour of GOD ring-disk electrodes, Albery and Bartlett (1985) have shown that the sensor response to low glucose concentration is limited by substrate diffusion through the covering membrane. In contrast to Kulys (1986) they postulated a direct electron transfer between enzyme and electrode surface on the grounds that (i) TCNQ is not soluble enough, and (ii) the reoxidation of GOD is too slow to explain the measured current as a result of dissolved TCNQ^- .

Later on, Albery et al. (1987) explained the experimental results by a mechanism that assumes only the adsorbed GOD molecules to be active, and omitted the covering membrane from the TCNQ electrode. Yet the functional stability of the sensor was high. This sensor type has been implanted in a rat brain and used continuously for glucose determination for ten days (Boutelle et al., 1986).

The principles outlined demonstrate that glucose sensors independent of soluble reagents may be realized by different techniques of enzyme and mediator immobilization. As mentioned in Section 2.2, direct electron transfer between the enzyme and the electrode is also possible. In this case the amperometric electrode replaces the cofactor or an oxidase. Wingard (1984) developed the concept of binding the prosthetic group of GOD directly to the electrode by a hydrocarbon chain having conjugated double bonds. Although in this way a FAD-modified electrode was obtained, recombination with apo-GOD did not give a glucose-oxidizing sensor. It is well known that only GOD consisting of two subunits, i.e. containing 2 FAD, is enzymatically active whereas the monomer does not catalyze glucose oxidation. Furthermore, as the FAD group is localized in a cavity 1.1–1.3 nm deep rather than at the molecule surface (Kulys and Cenas, 1983), it is unlikely that directly bound FAD without a long spacer may interact with apo-GOD to form native holoenzyme. The activity found in analogous experiments by Sonowat (1984) was probably due to artefacts such as recombination of dissociated FAD with adsorbed apo-GOD.

The direct electron transfer between GOD and an electrochemically pretreated platinum electrode has been studied by Durliat and Comtat (1984). Spectroelectrochemical investigations showed that GOD was quantitatively reduced at the electrode. A GOD solution (100 $\mu\text{mol/l}$) was entrapped in a reaction chamber of 0.04 mm thickness in front of the electrode by a dialysis membrane. The electrode was pretreated by cyclic sweeping between -700 mV and $+900$ mV for 8 h. In anaerobic solution this sensor responded to glucose at a potential of $+450$ mV with a response time of 6 min and a linear concentration range between 0.01 and 7 mmol/l.

A novel principle for accelerating the electron transfer has been the direct chemical modification of GOD by electron-mediating groups such as ferrocene derivatives (Heller and Degani, 1987). The distance between the mediator molecules was at most 1 nm and the 'relays' had to be attached in the vicinity of the prosthetic group. The binding of ferrocene to GOD was therefore conducted in 2 mol/l urea. After refold-

ing of the enzyme by removal of urea, 60% of the initial activity was still present. The functional stability was somewhat decreased by the chemical modification. A sensor using the modified GOD can be operated without additional reagents at an electrode potential of +200 mV vs SCE, i.e., on the basis of the anodic oxidation of the GOD-bound ferrocene.

In contrast to this approach to the introduction of electron relays in the interior of the enzyme molecule, Scheller et al. (1989b) suggested coupling benzoquinone (BQ) to the surface of the GOD molecule. About 15 BQ molecules could be bound to the NH_2 -lysine groups of the native enzyme. After adsorption of the modified enzyme at a carbon electrode both the BQ groups and FAD were visible in cyclic voltammograms as reflected by two distinct peaks. The peak separation of 20–30 mV and the $E_{1/2}$ of -370 mV indicated a rapid electron exchange of the prosthetic group. On addition of glucose the cathodic FAD peak disappeared whereas the reoxidation peak of FADH_2 was increased. Native GOD did not exhibit this catalytic current, so that the BQ modification was likely to be responsible for the fast electron transfer.

3.1.1.4 Biochemically Modified Electronic Devices

The increasing level of biosensor integration leads from the common arrangement with separate signal processing to the inclusion of the electronics within the sensor body. Therefore, in addition to reagentless microelectrodes modified by enzyme and mediator, the direct combination of electronic devices with enzymes has been achieved. This research centers on sensor miniaturization, multifunctional sensors, and the employment of integrated circuit technology. Structures containing several identical sensors or enzyme-free reference sensors and electronics for the statistical evaluation and elimination of interferences on the same chip have also been designed. Three basic types of electronic microsensors have been utilized so far:

- (i) amperometric microelectrodes (thin film sensors);
- (ii) ion selective field effect transistors (ISFETs);
- (iii) palladium- and/or iridium-sensitized metal oxide semiconductor (MOS) FETs for hydrogen and ammonia.

The latter direction was initiated by Danielsson et al. (1979), who combined immobilized hydrogenase with a hydrogen gas-sensing MOSFET. Since the MOSFET requires a high operational temperature it was separated from the enzyme layer. Caras and Janata (1980) directly integrated the microelectronic sensor and the immobilized

enzyme by fixing a layer of β -lactamase at the gate of a pH sensitive FET to design a penicillin sensor.

Whereas mass production of integrated circuits including ISFETs is an almost completely automated process, coupling of the enzyme is mostly performed by traditional methods, which is why the development of an enzyme FET technology compatible with that of integrated circuit production is of major importance. A main problem is the tight attachment of defined enzyme layers to the tiny gate regions. The operational conditions, e.g. temperature and solvent, have to be adjusted so as to avoid damage to the biocatalyst molecules. Common procedures in photolithographic technology such as spin coating, photopolymerization of positive or negative resists using optical masks, and developing of uncovered regions by organic solvents, have been realized, step by step, for the structuring of enzyme layers.

1. Amperometric Microelectrodes

A microbiosensor based on a Clark-type oxygen electrode has been described by Miyahara et al. (1983) (Fig. 51). The sensor was constructed by the anisotropic etching technique on a silicon substrate using two p-Si-chips of 300 μm thickness and an electric resistance of 3–5 Ωcm . The lower chip was masked by SiO_2 and anisotropically etched in KOH to create the internal electrolyte (1 mol/l KOH) reservoir and the cavity for the counter electrode. A gold cathode and a silver anode were formed by vacuum deposition. The upper Si-chip contained a window of 400 μm x 400 μm to limit the active surface of the working electrode. An oxygen-permeable Teflon membrane was introduced between the chips which were glued together and electrically insulated with epoxy resin. The sensor exhibited a response time of 12 s and a linear dependence on oxygen concentration. The measuring solution had to be stirred. The sensor was stable for about 100 h.

For glucose determination the part of the Teflon membrane covering the Au cathode was covered by a cellulose membrane containing GOD immobilized by glutaraldehyde crosslinking. The response time for glucose was 5–10 min and a linear concentration dependence was obtained between 0.1 and 1 mmol/l.

A glucose microsensor based on difference measurement between two gold microelectrodes, one covered by native and one by denatured GOD, has been described by Takatsu and Morizuma (1987). This combination permits the elimination of electrochemical interferences without resorting the use of permselective membranes. The gold electrodes were

evaporated on a chromium-coated glass support (Fig. 52) and the enzyme was immobilized in a photosensitive poly(vinyl acetate) film, i.e., only microelectronic fabrication technology was used. This procedure was claimed to be cheap enough to render the sensor a disposable material for home diagnostics. The linear measuring range was up to 5 mmol/l glucose; after 100 measurements 90% of the initial activity was still present.

Hintsche et al. (1989) developed an amperometric glucose sensor having excellent analytical characteristics. The base sensor was fabricated by thermal or electron beam evaporation of titanium, gold, and platinum and deposition on silicon. GOD was entrapped on the electrode in polyurethane crosslinked at the surface by polyisocyanate treatment. The anodic oxidation of hydrogen peroxide at the sensor reached the limiting current at + 0.6 V. The response time for glucose was about 1 s and with intermittent use the lifetime of the sensor exceeded one year. Microbiosensors for glucose and glutamate based on H_2O_2 - and O_2 -sensing microelectrodes have been developed by Karube and Tamiya (1986). The base sensors were assembled by vacuum evaporation on a Si_3N_4 support. The electric connections were insulated by Ta_2O_5 . The gold electrode, polarized to +1.1 V for H_2O_2 detection, was covered with GOD by dipping it into a solution containing enzyme, BSA, and glutaraldehyde. The response time of this sensor for glucose was 2 min and the calibration curve was linear up to 0.5 mmol/l. For use with the O_2 -indicating electrode the enzyme was bound to a cellulose triacetate membrane.

A planar glucose sensor using an ISFET in place of the reference electrode has been introduced by the Japanese NEC Corporation (Mura-

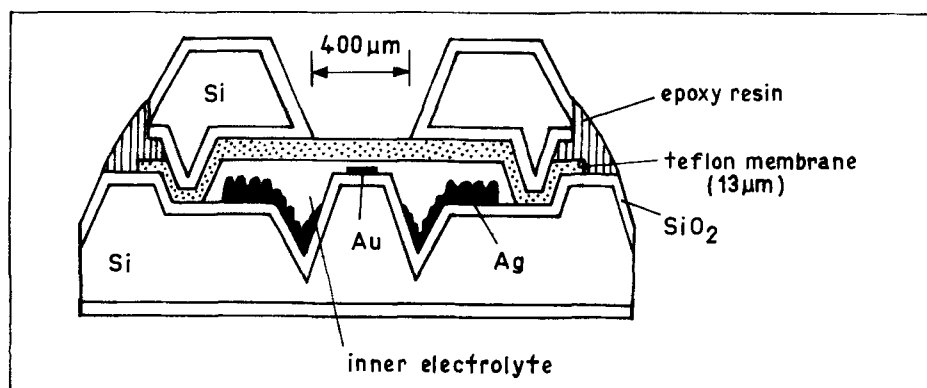


Fig. 51. Structure of an oxygen microelectrode. (Redrawn from Miyahara et al., 1983).

kami et al., 1986). A gold layer of 1 μm thickness was sputtered on a titanium-coated sapphire support. Two working electrodes (0.4 mm x 2.3 mm) were created by structuring with negative photoresist and etching; the bonds were insulated by means of a photoresist. To cover the gold electrode with enzyme the wafer was coated by a positive resist. After developing the area of the working electrode, this surface was silanized, treated with glutaraldehyde, and covered with a solution of GOD and BSA in 5% glutaraldehyde. After drying, the photoresist was removed by sonication of the wafer in acetone. The working and counter electrode were connected to a potentiostat using the ISFET as reference electrode. The sensor exhibited the same current-potential curves for H_2O_2 as did a calomel reference electrode. A response time for glucose of only 10 s and a linear range up to 5 mmol/l were obtained. The sensor is advantageous in that all parts can be produced by integrated circuit technology.

The fixation of enzymes to plasma polymers formed by electric discharge reactions (plasma polymerization) in vacuum devices has been achieved by Kampfrath et al. (1990). The enzyme was coupled via glutaraldehyde or carbodiimide by using amino or carboxyl group-carrying organic substances and subsequently treated with NH_3 or O_2 plasma. Application of masks in the polymer deposition process permitted the creation of structured coverage of the electrode surfaces. The method gave rapidly-responding glucose sensors with apparent GOD activities up to 200 mU/cm^2 .

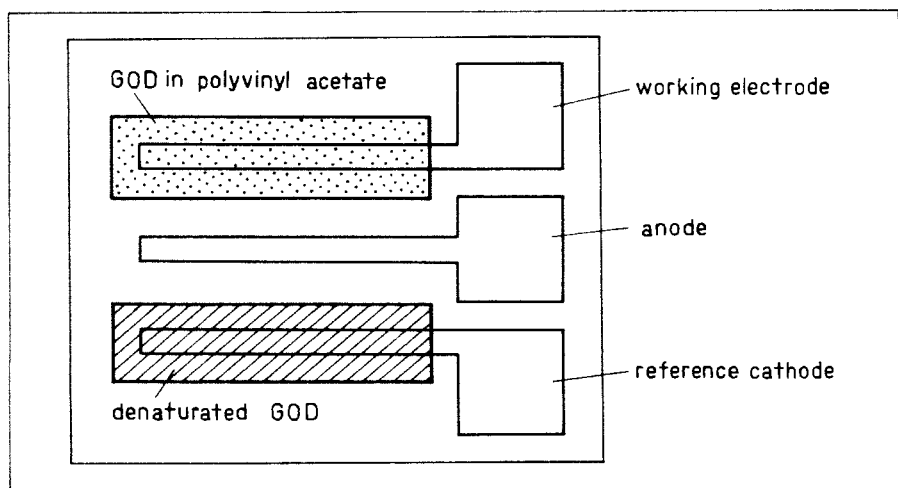


Fig. 52. Thin film electrode configuration of a disposable glucose sensor. (Redrawn from Morizuma et al., 1986).

Foulds and Lowe (1986) combined mass production of the base sensor and enzyme immobilization as follows. Using gold or platinum ink, a working and counter electrode were deposited on a ceramic substrate. After thermal treatment of the electrode material a solution containing GOD and a pyrrole derivative of ferrocene was electrochemically polymerized at the electrode. The pyrrole component forms a conducting polymer and the immobilized ferrocene acts as electron acceptor for GOD. The structured immobilization permits this technique to be used for successive enzyme fixation to multiparameter sensors.

2. Enzyme Field Effect Transistors

Concurrently with the first amperometric microbiosensor for glucose, Hanazato and Shiono (1983) described a potentiometric glucose oxidase FET. Two ISFETs (Si chips, 0.6 mm x 6.5 mm) and a Pt pseudo-reference electrode were encapsulated with epoxy resin on an epoxy support. The gate region of one of the FETs was covered by a solution of GOD and BSA in a photosensitive polymer. Excess enzyme was removed by centrifugation and the remaining layer polymerized by light irradiation and crosslinked by glutaraldehyde. Since the pH signal was light sensitive, the GOD chip had to be tested in the dark. In order to eliminate pH effects of the sample, a difference circuit was used (Fig. 53). The time needed to achieve a steady signal was 9 min; 95% of the final value were reached within 3 min. The signal was linearly (not, as expected, logarithmically) dependent on glucose concentration up to 15 mmol/l.

The same research group (Shiono et al., 1986, 1987) further improved the enzyme immobilization technique as follows. GOD and lipase were mixed with BSA, poly(vinylpyrrolidone), and a photosensitive bisazido compound and transferred to the silanized FET surface. The gate region was irradiated with ultraviolet light, the untreated region removed by development by water, and the remaining enzyme film crosslinked with glutaraldehyde. This photolithographic procedure resulted in a two-parameter FET bioprobe for glucose and trioleate determination in diluted blood serum. The sensitivity to glucose was rather poor; 0.6 mmol/l glucose caused a signal of only 7 mV. This sensor type is intended for commercialization by Mitsubishi Electric Corporation (Japan).

Honold and Cammann (1987) covered a pH sensitive Ta₂O₅ gate of an FET structure with GOD and used a reference gate to eliminate the influence of pH variations in the measuring solution. Entrapment of GOD together with catalase in a relatively thick PVC layer gave a sensor responding to glucose up to 110 mmol/l with a sensitivity of 12 mV per

decade. After 100 days of continuous contact with glucose the sensitivity was still 50%. When GOD was crosslinked with glutaraldehyde only, without catalase, the sensitivity was increased by a factor of 10 but the upper limit of linearity decreased to 0.5 mmol/l.

Caras and Janata (1985) described the use of two pH-FETs and two MOSFETs on one chip for glucose determination. One pH-FET was covered with polyacrylamide gel containing GOD and catalase, the other with gel with no enzyme. In order to obtain a defined layer thickness a laminated photoresist of 50 μm thickness was deposited on the chip, leaving the gates uncovered. The polyacrylamide gel was dropped into the pools on the gates and polymerized. Adhesion of the enzymes was improved by pretreatment with BSA and crosslinking with glutaraldehyde. The response time of the glucose FET was 2 min and the semilogarithmic calibration curve was linear between 1 and 10 mmol/l glucose.

Miyahara et al. (1985) developed an integrated enzyme FET based on a silicon-on-sapphire (SOS) sensor for simultaneous determination of glucose and urea. Three ISFETs and two metal insulator semiconductor FETs (MISFETs) were integrated on a surface area of 2.5 mm x 2.5 mm (Fig. 54). One of the ISFETs served as reference sensor in order to compensate the signals caused by pH changes of the solution; the two others were covered by GOD and urease, respectively. The MISFETs can be used as pH electrodes. For enzyme immobilization the chip was covered with a laminated photosensitive layer of 75 μm thickness and,

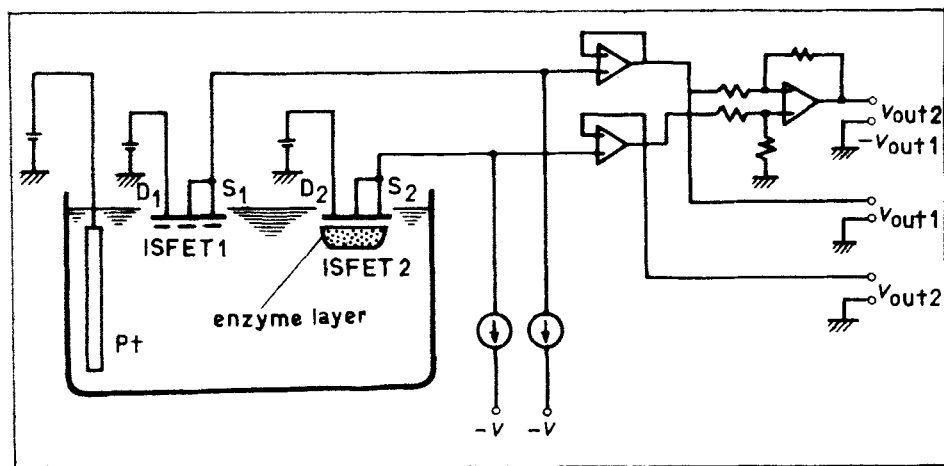


Fig. 53. Differential circuit of two ISFETs. (Redrawn from Hanazato and Shiono, 1983).

after development, the respective pools at the gate regions were formed. One pool was filled with 10% photosensitized poly(vinyl alcohol) containing 10 mg/ml GOD, the other with an analogous urease solution. The reference FET remained uncovered. After drying, the polyvinyl acetate film was crosslinked by UV irradiation. The chip was washed with phosphate buffer for 2 h before use. Owing to the poor adhesion of the enzyme membrane the sensor had a lifetime of only a few days. A higher stability could be obtained by pretreating the Si_3N_4 gate surface with γ -aminopropyl triethoxysilane and crosslinking the enzyme with glutaraldehyde. In unstirred solution the maximum glucose signal was attained 30 min after sample injection. The response time for urea was 90 s. For both substrates the measuring range was 0.1–10 mmol/l.

National Electric Company (Japan) developed an integrated biosensor based on an SOS-chip containing a K^+ FET and two pH FETs covered with GOD and urease, respectively (Kimura et al. 1985). The enzymes were immobilized by crosslinking with glutaraldehyde as described by Miyahara et al. (1985).

Alternatively, the above authors (Kimura et al., 1989) used an ink jet nozzle as a tool for precise enzyme deposition on a multigate ISFET.

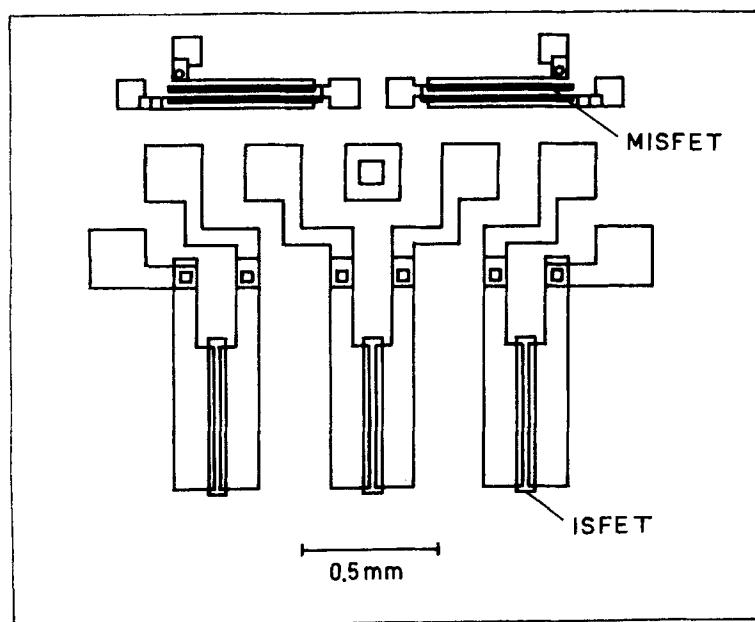


Fig. 54. Layout of an integrated ISFET-MISFET configuration. (Redrawn from Miyahara et al., 1985).

Drops of GOD or urease solution were deposited onto the gate regions using an X-Y stage controlled by a computer. Immobilization of the enzymes was achieved by keeping the ISFET in a chamber filled with glutaraldehyde vapor.

Extended gate structures using iridium oxide layers have been used in an integrated glucose and urea sensor (Araki et al., 1985).

Whenever pH alterations of the sample can be eliminated by two pH-ISFETs working in differential mode the buffer capacity of the sample solution influences the sensitivity of the enzyme-covered gate. To create an enzyme-FET that is independent of this parameter, Van der Schoot and Bergveld, (1987/88) developed a coulometric buffer compensation system. The operation of the system is such that the coulometric electrode inside the enzyme layer adjusts the pH of the enzyme-FET to that of the bulk as measured by the reference FET. Thus the enzyme operates at constant pH. The magnitude of the charge which is needed for this electrolytic proton generation is a measure of the substrate concentration. The authors called this arrangement a 'pH-static enzyme sensor'.

An alternative variant for the elimination of disturbances by buffer capacity variations is the coupling of peroxide-generating oxidases, such as GOD, with the peroxidase-catalyzed fluoride formation to fluoride sensitive FETs. Using GOD and HRP entrapped in polyurethane and pentafluorophenol as HRP cosubstrate, Dransfeld et al. (1990) obtained a slope of 41.3 mV per decade of glucose concentration with a lower detection limit of 0.01 mmol/l. This principle has been extended to the generation of the reference signal on the same chip. A bifunctional FET carrying gates for both H^+ and F^- and a gold auxiliary electrode was uniformly covered by the bienzyme layer. For highly buffered background solutions the difference signal reflects only the glucose concentration since changes of pH or buffer capacity are suppressed by proper dilution of the sample. It has, furthermore, been demonstrated that in the presence of 1 mmol/l fluoride (and in the absence of pentafluorophenol) the F^- sensitive gate can be used as a reference for the GOD-covered pH sensitive gate on the same FET. In both cases the difference signals exhibit the same concentration dependence as the respective monofunctional FET combined with an external calomel reference electrode.

The results obtained so far with biosensors based on biochemically modified small scale electronic devices demonstrate the possibility of producing multianalyte sensors by Si-chip technology. Although these

sensors exhibit good functional characteristics they are not yet suitable for practical routine application. In addition to the low lifetime and the long response time, the main obstacle is the susceptibility of the sensors to disturbances by solution properties such as varying oxygen tension or pH. These problems have been largely overcome for reagentless sensors based on modified electrodes. Furthermore, reliable electronic signal processing within the sensor itself has not been realized as yet. Commercialization of enzyme FETs, although announced as early as 1984 (Schmid, 1985), has not yet succeeded. At present, disposable sensors combined with miniature electronic units appear more promising. Among these, thin film electrodes seem to be best suited for large scale application.

3.1.2 Galactose Sensors

Galactose is oxidized under the formation of hydrogen peroxide by the copper enzyme galactose oxidase (EC 1.1.3.9). Galactose oxidase is not as highly specific as glucose oxidase. Many other substrates react with the enzyme, including lactose, glycerol, dihydroxyacetone, and glyceraldehyde.

The principle of potential-dependent selectivity control of a galactose oxidase membrane (Johnson et al., 1982, 1985) using galactose, raffinose, and dihydroxyacetone as substrates has been discussed in Section 2.2.2. The change of the redox potential influences all reactions in the same direction. However, since the activity is different towards different substrates the degree of conversion of a particular substrate can be affected by the applied potential. At first the better substrates, e.g. raffinose or galactose, can be measured and at optimal potential all substrates are detected.

Traylor et al. (1977) immobilized galactose oxidase by glutaraldehyde crosslinking to an asymmetric cellulose acetate membrane of 1 μm thickness and 0.6 mm pore diameter. This relatively unstable layer was covered by a polycarbonate membrane having pores of 30 nm diameter. The enzyme was maintained in an oxidized state by addition of ferricyanide to avoid the effects of reducing substances present in serum. Enzyme inactivation was reduced by Cu^{2+} . The sensor responded linearly to galactose in the range 0–25 mmol/l. The only physiologically important interferent was dihydroxyacetone.

Lang et al. (1983) devised a microelectrode for galactose determination in tissue. In analogy to the respective glucose sensor, Dicks et al.

(1986) developed a galactose probe based on an electrode modified with 1,1'-dimethylferrocene. Galactose oxidase was entrapped in front of the electrode by a dialysis membrane.

3.1.3 Enzyme Electrodes for Gluconate

Gluconate is used to an increasing extent in the food industry as flavoring substance. Miki et al. (1985) entrapped gluconate dehydrogenase (EC 1.1.99.3) from *Pseudomonas fluorescens* on the surface of a benzoquinone-carbon paste electrode by means of a dialysis membrane. With a response time of 1 min a linear dependence of the current at +300 mV vs SCE up to 3 mmol/l gluconate was obtained. Ikeda et al. (1987, 1988a) conducted a study for other mediators and found ferricyanide, dichlorophenol indophenol, and coenzyme Q₇ to be active with gluconate dehydrogenase.

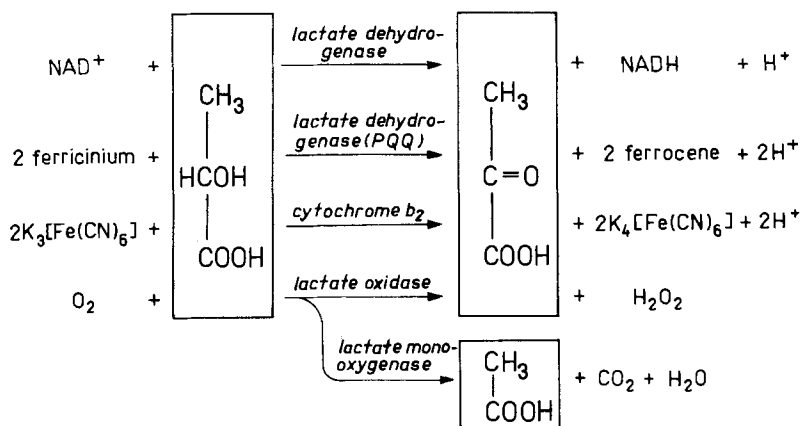
Without external electron mediators a catalytic current of gluconate was observed at a carbon electrode bearing irreversibly adsorbed gluconate dehydrogenase (Ikeda et al., 1988b). Obviously, the substrate was oxidized via the electron transfer from the gluconate-binding FAD and the heme groups to the electrode. Although this bioelectrocatalytic sensor was relatively unstable and insensitive, it demonstrated the analytical applicability of mediatorless electron transfer.

3.1.4 Lactate Sensors

L-Lactate is an intermediate product of carbohydrate metabolism. An increase in blood lactate concentration caused by oxygen deprivation of tissues and liver disease makes lactate a relevant indicator in clinical and sports medicine. The normal concentration in blood is up to 2.7 mmol/l; strenuous exercise may raise it to 25 mmol/l. Lactate determination is also of interest in the food and microbiological industries.

Lactate is usually determined by photometric detection of NADH formed in the reaction catalyzed by lactate dehydrogenase (LDH, EC 1.1.1.27). The pH optimum for the forward reaction of LDH (MW 135 000) is about 9, the K_M for lactate 6.7 mmol/l. Since the equilibrium lies far to the left ($K = 2.76 \cdot 10^{-5}$ mol/l at pH 7.0), hydrazine, pyruvate oxidase, or alanine aminotransferase have to be added to trap the pyruvate formed.

The enzyme reactions applicable in biosensors for lactate determination are shown in the following scheme:



Besides sensors using isolated enzymes, cell-based lactate sensors using cytochrome b_2 -containing yeast (*Hansenula anomala*, *Saccharomyces cerevisiae*) (Kulys and Kadziauskiene, 1978; Vincké et al., 1985a; Hauptmann, 1985; Racek and Musil, 1987) and erythrocytes (Racek, 1987) have also been proposed.

Lactate monooxygenase (LMO, EC 1.13.12.4) from *Mycobacterium smegmatis* is a flavoprotein with a molecular weight of 340 000 and a $K_M(\text{lactate})$ of 8 mmol/l. The enzyme has been immobilized on porous glass and employed in an enzyme thermistor allowing lactate to be measured in the range 0.005–2 mmol/l (Danielsson et al., 1981).

Schelter-Graf et al. (1984) coupled an LDH column reactor with electrochemical NADH indication. These authors increased the electrochemical selectivity by using a graphite electrode modified with 3- β -naphthoyl-Nile Blue which oxidizes NADH at a potential of -0.22 V vs SCE. In an FIA system a sample frequency of 15/h with a relative standard deviation of 1% was obtained.

The different cosubstrate specificities of the lactate-oxidizing enzymes offer the use of a great variety of electrochemical indicator reactions in membrane sensors. In enzyme electrodes based on LDH the biochemical reaction has been coupled to the electrode via NADH oxidation, either directly or by using mediators or additional enzymes (see Section 3.2.1). This leads to a shift of the unfavorable reaction equilibrium by partial trapping of the reduced cofactor. Such a shift has also been achieved by using pyruvate oxidase coimmobilized with LDH (Mizutani, 1982).

Blaedel and Jenkins (1976) evaluated two LDH–NAD⁺ combinations for a reagentless lactate sensor (Fig. 55). NAD⁺ was either coimmobilized with LDH to cellulose or an NAD⁺-agarose complex was constrained together with LDH to a region near the electrode surface. By bringing the bound NAD⁺ into intimate physical contact with the electrode, the immobilized cofactor was recycled electrochemically and reused by the enzyme. This eliminated the need to supply NAD⁺ as a reagent.

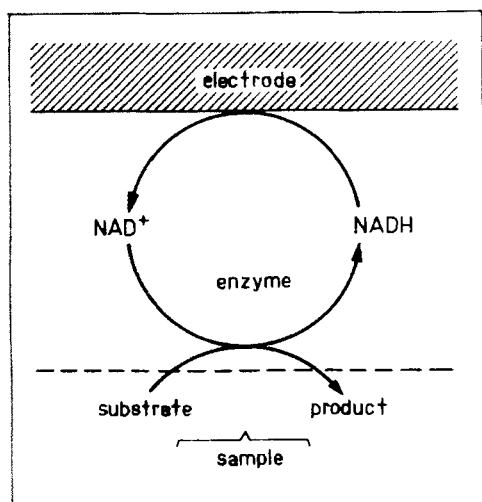
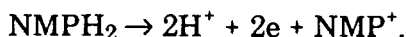


Fig. 55. Schematic representation of a reagentless enzyme electrode using electrochemical regeneration of NAD⁺ for the determination of reduced dehydrogenase substrates.

For anodic NADH oxidation a potential of more than +0.4 V is necessary. Since this high overpotential favors electrochemical interferences, various investigations have been conducted to decrease the oxidation potential by using mediators or pretreating the electrode. Cenas et al. (1984) found that after electrochemical pretreatment of glassy carbon electrodes in the range of –0.8–1.8 V, NADH oxidation occurs at 0–0.2 V vs Ag/AgCl. LDH was entrapped on top of the electrode by means of a dialysis membrane. The oxidation current was proportional to lactate concentration up to 10 mmol/l. Presumably because of adsorption of NADH oxidation products the half life of the sensor was less than 3 days.

The mediator NMP⁺ has been used for lactate determination with a platinum electrode in front of which LDH and dextran-bound NAD⁺

were fixed by a semipermeable membrane (Malinauskas and Kulys, 1978). The measuring signal is formed by reoxidation at the electrode of the mediator reduced by NADH:



The response time of the sensor has thus been lowered by 50% as compared with direct oxidation of NADH.

Chen and Liu (1977) utilized the spontaneous oxidation of NADH by potassium ferricyanide for the construction of a potentiometric LDH electrode. The coupled reduction of ferricyanide ions to ferrocyanide ions results in a measurable electrochemical zero-current potential. The potential was found to be Nernstian in nature and directly proportional to the logarithm values of lactate concentration over the range 0.02 to 50 mmol/l. The response time was as high as 10 min.

LDH(PQQ) is a quinoprotein requiring neither oxygen nor nicotinamide cofactors for lactate oxidation. The enzyme has been coupled to a ferrocene-modified carbon electrode (Turner, 1985). The sensor was useful for lactate determination up to 4 mmol/l but within 5 h the sensitivity dropped to 5%.

Cytochrome b_2 (EC 1.1.2.3) is a tetrameric protein in which each subunit contains one molecule of flavin mononucleotide and one molecule of heme. The molecular weight is 238 000. The enzyme is contained in *Saccharomyces cerevisiae* and *Hansenula anomala*, where it transfers electrons in the respiratory chain from lactate to cytochrome c. With the artificial electron acceptor hexacyanoferrate(III) the respective K_M values are 0.4 and 1.3 mmol/l. The pH optimum is between 6.5 and 8.

In most amperometric cytochrome b_2 electrodes the reaction is followed by anodic oxidation of ferrocyanide at a potential of +0.25 V or above. The first of such sensors was assembled by Williams et al. (1970), who immobilized the enzyme (from baker's yeast) physically at the tip of a platinum electrode within a nylon net of 0.15 mm thickness. The large layer thickness resulted in a response time of 3–10 min. Owing to the low specific enzyme activity used, the sensor was kinetically controlled. Therefore the linear measuring range extended only up to 0.1 K_M . A similar sensor has been applied by Durliat et al. (1979) to continuous lactate analysis. The enzyme was contained in a reaction chamber of 1 μl volume in front of the electrode. This principle has also been employed in the first commercial lactate analyzer using an enzyme electrode (Roche LA 640, see Section 5.2.3.3). With a sensor stability of 30 days and a CV below 5%, 20–30 samples/h can be processed with this device.

Cytochrome b_2 from *Hansenula anomala* has been employed for lactate determination in the 'Glukometer GKM 02' analyzer (ZWG, GDR). When immobilized in gelatin or poly(vinyl alcohol) the enzyme was stable for 15 days. Linearity was obtained over the range 10 $\mu\text{mol/l}$ –2.4 mmol/l, the upper limit being adjustable by the mediator concentration used. With the rate method a sample frequency of 40/h and a CV below 2% were achieved. In a flow-through device the CV was below 1% (Schubert and Weigelt, 1986).

In order to fabricate reagentless sensors, Kulys and Svirnickas (1980b) coimmobilized cytochrome b_2 together with semiconductive organic metal complexes ($\text{NMP}^+\text{TCNQ}^-$) by physical entrapment on the tip of a Pt probe. The charge transfer complex acts as a mediator and permits lactate to be measured at a potential between -0.03 and $+0.4$ V. Oxygen decreased the electrode sensitivity by 50% by spontaneous reaction with the reduced organometallic compounds.

Another reagentless sensor was studied by Durliat and Comtat (1980), who employed coimmobilized cytochrome b_2 and the physiological electron acceptor of the enzyme, cytochrome c. The ferrocytochrome c formed was reoxidized at $+500$ mV. The independence of the sensor from external mediators was considered to be an important precondition for *in vivo* application.

Shinbo et al. (1979) developed a potentiometric cytochrome b_2 electrode. The change of the redox ratio of ferrocyanide/ferricyanide was indicated. The plot of the potential change versus the logarithm of lactate concentration yielded an S-shaped curve.

The reaction of LMO can be followed amperometrically by using a Clark-type oxygen electrode. This combination was first described by Schindler and Von Gülich (1981). Later on, Mascini et al. (1984) and Weaver and Vадgama (1986) employed LMO in sensors for lactate determination in serum, plasma, and whole blood. The latter authors entrapped the enzyme in gelatin and polyacrylamide, as well as ultrafiltration membranes. Regardless of the immobilization method used, the response time of the respective sensors was 3 to 4 min. The sensitivity and stability increased in the order polyacrylamide < gelatin < ultrafiltration membrane. A linear range of 0.008–0.8 mmol/l lactate was obtained in a flow-through apparatus containing a sensor furnished with the latter membrane type.

Weigelt et al. (1987a) optimized an LMO-gelatin membrane for application in the 'Glukometer' analyzer (ZWG, GDR). The enzyme loading test (Fig. 56) shows that above 1 U/cm^2 the sensor is diffusion controlled,

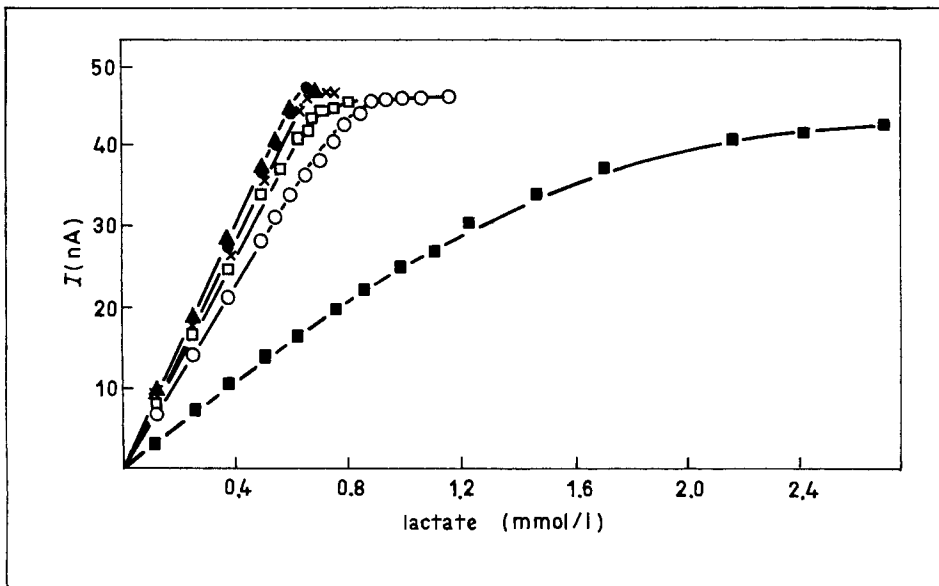


Fig. 56. Influence of the enzyme loading of a LMO sensor on the dependent of the current on lactate concentration. ■: 0.1 U/cm², ○: 0.5 U/cm², □: 1.0 U/cm², ×: 2.5 U/cm², ●: 10.0 U/cm², ▲: 15.0 U/cm². (Redrawn from Weigelt et al., 1987a).

i.e., the substrate is completely oxidized within the membrane. In order to increase the lifetime a tenfold excess of enzyme was routinely used, allowing application of the sensor for 55 days or 600 determinations. 60 Measurements may be performed per hour. The CV was found to be 1%. As can be judged from the sharp leveling off of the curves in Fig. 56, the upper limit of linearity was set by oxygen limitation.

The LMO sensor has also been applied to the sequential determination of lactate and LDH activity. When the current decrease resulting from lactate oxidation was complete, NADH and pyruvate were added to the measuring cell. The subsequent current decrease reflects the lactate formation by the LDH-catalyzed reaction. The time required for one sequential measurement was 4 min. The CV for 20 LDH determinations was 1.2%.

The flavoprotein lactate oxidase (LOD, EC 1.1.3.2, MW 80 000) has a K_M for lactate of 0.7 mmol/l and is suited for coupling with oxygen as well as hydrogen peroxide electrodes. Both variants have been investigated. Mizutani et al. (1983) described an electrode based on immobilized LOD for the sequential determination of lactate and LDH. In analogy to the relevant LMO sensor (Weigelt et al., 1987a), an oxygen

probe was used, since the NADH required for the LDH reaction is also oxidizable at the potential of anodic H_2O_2 oxidation. The time needed to accomplish one sequential measurement of lactate (0.005–0.5 mmol/l) and LDH (1–300 U/l) was 7 min. The sensor was stable for more than 2 weeks or 140 samples.

Polyurethane membranes containing immobilized LOD are being applied to lactate determination in the Glukometer analyzer. Similar to GOD, a sample frequency of 60/h and high precision ($\text{CV} < 3\%$) were obtained. The linear range was 0.01–0.5 mmol/l. Other self-contained lactate analyzers such as those from Yellow Springs Instrument Co. (USA) and Omron Tateisi (Japan) also incorporate LOD sensors (see Section 5.2.3.3).

Using an LOD sensor, Bardeletti et al. (1986) achieved a linear concentration dependence for lactate between 0.25 and 250 $\mu\text{mol/l}$. This unusually high sensitivity was obtained by immobilization of the enzyme on an immunoaffinity membrane such as is commercially available for antibody fixation (Pall Biodyne, USA). The large pores of this membrane provide very fast substrate and product diffusion. For LOD binding the membrane was simply dipped into an enzyme solution.

Mullen et al. (1986) decreased the permeability of LOD membranes in order to extend the linear range to higher lactate concentration. A polycarbonate membrane was treated with methyltrichlorosilane prior to enzyme immobilization by crosslinking with glutaraldehyde and BSA. In this way the upper limit of linearity was shifted from 0.2 to 18 mmol/l. The permeation of electrochemical interferents was diminished concomitantly. On the other hand, the silanization increased the response time from 0.5–1 min to 1–3 min and reduced the sensitivity by 98–99%.

Scheller et al. (1986a) combined polyurethane-immobilized LOD with an Au/Pd-sputtered carbon electrode. The electrode modification permits H_2O_2 to be electrochemically oxidized at a potential as low as +450 mV, where interferences by other anodically oxidizable compounds such as NADH and ascorbic acid are largely reduced (Fig. 57). This increased selectivity also enables the measurement of LDH activity. The sensor has been introduced in an FIA manifold. A sample frequency of 200/h (Fig. 58) and a CV below 1% were obtained with this setup. A platinum electrode with LOD covalently bound to a nylon membrane has been employed for continuous blood lactate determination in an artificial pancreas by Mascini et al. (1985b, 1987).

Attempts to construct lactate sensors by biochemical modification of electrode surfaces have been made using LDH as well as cytochrome b_2 .

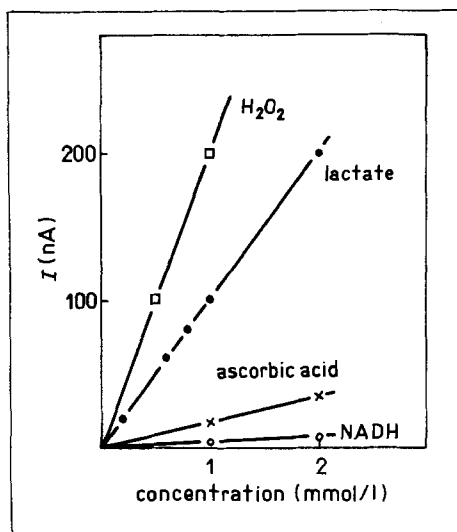


Fig. 57. Concentration dependence of the peak current of an FIA-integrated LOD electrode at injection of 40 μ l of L-lactate, H₂O₂, and ascorbic acid. (Redrawn from Scheller et al., 1986a).

Laval et al. (1984) bound LDH covalently to electrochemically pretreated carbon. The enzyme was fixed by carbodiimide coupling simultaneously with anodic oxidation of the electrode surface. The total amount of immobilized LDH was determined fluorimetrically after removal from the electrode and hydrolysis. The authors found that at a maximal enzyme loading of 13 pmol/cm² six enzyme layers are formed. The immobilization yield was about 15%. The kinetic constants, v_{\max} and K_M , were not affected by the immobilization. The obtained enzyme loading factor of 10^{-3} indicates that diffusion in the enzyme layer was of minor influence on the response of the sensor. The layer behaved like a kinetically controlled enzyme membrane, i.e., the NADH oxidation current was proportional to the substrate concentration only far below K_M . With increasing enzyme loading the sensitivity for NADH decreased due to masking of the electrode surface.

Yao and Musha (1979) modified an n-octaldehyde-containing carbon paste electrode with NAD⁺. LDH was added to the measuring solution. The NADH formed upon lactate injection was oxidized electrochemically, giving a well-defined, linear-sweep voltammetric peak. The peak area was linearly related to the concentration of lactate in the range 0.025–1.0 μ mol/l. Durliat and Comtat (1978) adsorbed cytochrome b₂ on

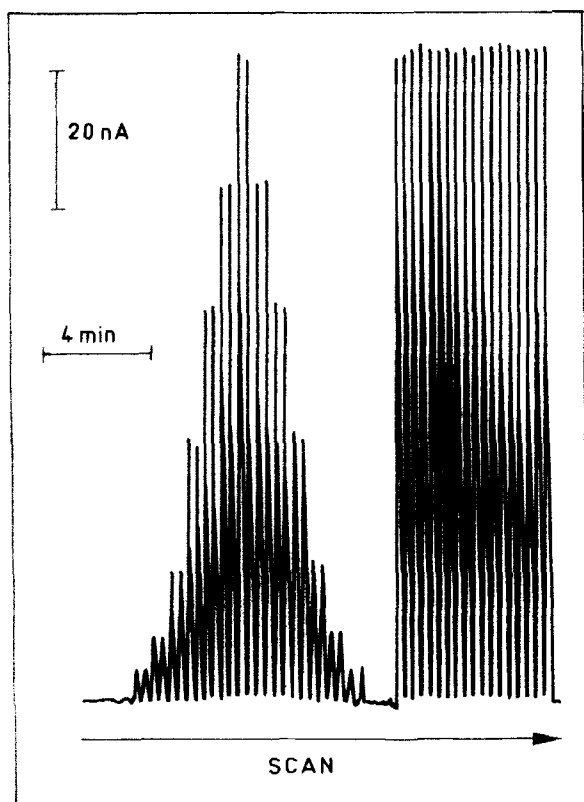


Fig. 58. Response curve of the LOD electrode as in Fig. 57 on injection of lactate standard solutions of 50, 100, 200, 400, 600, 800, and 1000 $\mu\text{mol/l}$. (Redrawn from Scheller et al., 1986a).

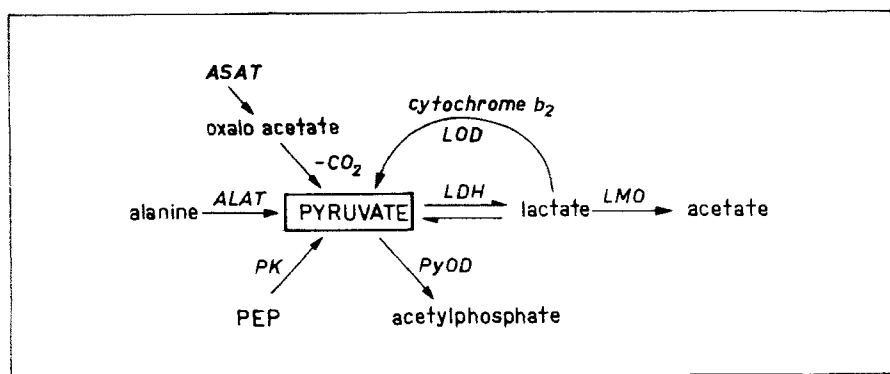


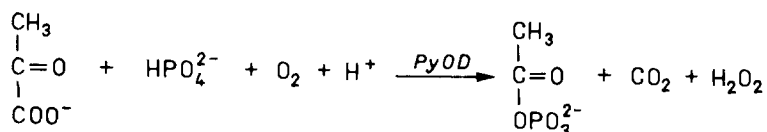
Fig. 59. Analytically utilized reactions involving pyruvate. PEP: phosphoenolpyruvate, PK: pyruvate kinase, ASAT: aspartate aminotransferase, ALAT: alanine aminotransferase.

a platinum electrode and found that the enzyme retained its activity in the adsorbed state.

3.1.5 Pyruvate Sensors

Pyruvate is a key intermediate in a pathway leading to acetyl-coenzyme A. The normal pyruvate concentration in blood serum is 0.04–0.12 mmol/l. Its determination is important because a considerable number of relevant metabolites and enzyme activities can be traced back to pyruvate (Fig. 59).

Pyruvate can be assayed by using the reactions catalyzed by lactate dehydrogenase and pyruvate oxidase (PyOD, EC 1.2.3.3):



Pyruvate oxidase requires the presence of thiamine pyrophosphate (0.1 mmol/l) and Ca^{2+} (2.5 mmol/l) for maximum activity. It should be used in 40 mmol/l Tris buffer, pH 6.5–7.5, containing 0.5 mmol/l phosphate. At higher phosphate concentrations substrate inhibition occurs; this effect has been utilized in a phosphate sensor based on immobilized PyOD (Tabata and Murachi, 1983). Since PyOD is relatively unstable, for biosensors the enzyme has been immobilized by physical entrapment in, e.g., collagen (Mizutani et al., 1980), poly(vinyl chloride) and acetylcellulose (Kihara et al., 1984a,b).

Mascini and Mazzei (1986) succeeded in the covalent immobilization of PyOD to a Biodyne Immunoaffinity Membrane (Pall Biodyne, USA) containing carboxylic groups on the surface. The membrane was preactivated with a carbodiimide derivative. The enzyme membrane was fixed on the tip of a hydrogen peroxide-indicating Pt electrode between a cellulose acetate membrane and a further dialysis membrane. This pyruvate sensor has been applied to serum measurement. Over 30 days the sensitivity dropped by only 13%.

The LDH sensors described in Section 3.1.4 can also be used for pyruvate measurement. Since the equilibrium of the LDH reaction lies far to the product side no trapping of lactate or NAD^+ is necessary. On the other hand, no indicator reactions that consume NADH within the enzyme layer can be applied, such as reaction with NMP^+ or HRP. However, some pyruvate sensors utilizing the signal formed by anodic

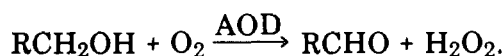
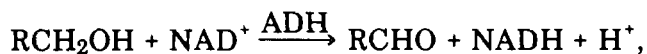
NADH oxidation have been described. Strnad (1989) adsorbed LDH on a carbon electrode sputtered with a mixture of gold and palladium. At this electrode NADH is oxidized at potentials as low as +0.6 V. The adsorbed enzyme was sufficiently active for the determination of millimolar pyruvate concentrations during 7 days.

Suaud-Chagny and Goup (1986) immobilized LDH on a pyrolytic carbon fiber microelectrode by impregnation in an inert protein sheath that was first electrochemically deposited around the active tip of the electrode. The NADH detection was improved by electrochemical treatment of the electrode. The detection limit for pyruvate was lower than 1 $\mu\text{mol/l}$. The sensor was used to estimate pyruvate concentration in rat cerebrospinal fluid.

3.1.6 Determination of Alcohols

Ethanol is the most common toxic substance involved in medical-legal cases. It is frequently a contributory factor in accidents. Methanol content is controlled in drinking water and fermentation media. It is also a widely used solvent in paints. The determination of other aliphatic alcohols is of minor importance.

For enzymatic alcohol measurement, alcohol dehydrogenase (ADH, EC 1.1.3.13) and alcohol oxidase (AOD, EC 1.11.1.6) from various sources have been used:



Because of the analogy of the AOD reaction to that of GOD, Clark in 1972 had already suggested an alcohol sensor based on AOD. Guilbault and Nanjo (1975a) described an H_2O_2 -sensing AOD electrode with a detection limit of 0.01 mg/ml.

The substrate specificity of AOD from different microbial sources has been studied in detail. Yeasts cultivated on methanol produce large amounts of AOD together with catalase. This AOD has a high activity towards methanol. Ethanol is oxidized more rapidly only by AOD from Basidiomycetes. AOD also reacts with other aliphatic alcohols, mercaptans, and formaldehyde. Acetone, isopropanol, lactate, and other hydroxyacids do not react. Because of the high catalase content of the

enzyme, most alcohol sensors are based on O_2 indication (Guilbault et al., 1983; Hopkins, 1985). Therefore, in solutions with varying oxygen concentration, a correction by an oxygen probe is required (Verduyn et al., 1983, 1984). For the above-mentioned sensors AOD was immobilized on pig small intestine and nylon membranes, respectively. The sensors equipped with these membranes were stable for 100–400 measurements.

Transcutaneous determination of ethanol with an oxygen electrode covered by AOD has been described by Clark (1979). Stepwise increases of the ethanol concentration in rat blood resulted in a curve reflecting the ethanol injections, returning to the initial value only after several hours. Disturbances were caused by variations in body temperature and blood pressure. In the paper cited, Clark developed the concept of a sensor for volatile enzyme substrates.

An alcohol electrode incorporating AOD immobilized in a hydrogen peroxide–selective sandwich membrane is commercially available from Yellow Springs Instrument Co. (USA).

AOD has been coimmobilized with alcohol dehydrogenase in order to increase the sensitivity of alcohol determination (Hopkins, 1985). In the presence of oxygen and NADH ethanol is recycled between the two enzymes. Thus more H_2O_2 is formed than substrate is present in the enzyme membrane, i.e., the sensitivity is enhanced (see also Section 3.2.4). A further advantage of this system is that this recycling is restricted to ethanol, because methanol is converted only by AOD but not by ADH. Conversely, isopropanol is oxidized by ADH but not by AOD. Thus, by combination of the two enzymes the selectivity of the sensor for ethanol is improved.

Aston et al. (1984) devised a methanol sensor for use in drinking water treated by methylotrophic bacteria for nitrate removal. Methanol oxidase from *Methylosinus trichosporium* was immobilized on a carbon paste electrode containing 1,1'-dimethylferrocene. The measured signal was linearly dependent on methanol concentration in the range of 1 to 5 mg/l. The intermediate product of methanol oxidation to formate (formaldehyde) was indicated with half the sensitivity. The sensor was stable for only 3 h.

The cofactor of ADH, NAD^+ , may not be replaced by other electron acceptors. Malinauskas and Kulys (1978) attempted to construct a reagentless alcohol sensor by coimmobilizing ADH with dextran-bound NAD^+ by a dialysis membrane in front of an oxygen electrode. The O_2 consumption was indicated via the reoxidation of NADH by NMP^+ . The system has also been used to measure NAD^+ with high sensitivity. In

the presence of an excess of ethanol and NMP^+ the cofactor is continuously recycled in a process consuming a large amount of oxygen. For this enzymatic-chemical recycling an amplification factor of 1000 was established (Kulys and Malinauskas, 1979a,b).

Torstensson et al. (1980) attached the cofactor directly to the active center of ADH in order to further integrate the reaction components in an alcohol sensor. During substrate conversion the cofactor was regenerated by anodic oxidation at a carbon electrode. Yao and Musha (1979) immobilized NAD^+ covalently on the carbon surface. ADH reacts with the immobilized cofactor and electrons are shuttled to the electrode by a mediator.

Mediator-chemically modified electrodes have been coupled either with ADH membranes to give enzyme electrodes (Cenas et al. 1984) or with ADH reactors, e.g., in an FIA device (Huck et al. 1984). Quinoidic groups, Meldola's Blue, and Nile Blue have been used as mediators. Alberty et al. (1987b) employed an electrode containing NMP^+ and TCNQ^- in a PVC carrier for NADH oxidation. ADH was entrapped on the sensor surface by a dialysis membrane.

A magnetic enzyme stirrer coupled with ADH immobilized on cellulose has been developed by Kuan et al. (1978). The immobilized enzyme was stable for 8 days. The reaction was indicated fluorimetrically.

Pyridine nucleotide-independent ADH from *Pseudomonas putida* has been applied in a sensor for aliphatic alcohols with a chain length of preferably C_6 to C_{10} (Vorberg and Schöpp, 1985). NMP^+ was used as electron acceptor and the O_2 consumption was indicated.

Arnold et al. (1987) described an optoelectronic ethanol sensor based on fluorimetric detection of NADH formed in the reaction catalyzed by ADH. The enzyme was fixed to the inner surface of a membrane permeable to volatile substances, which separated the sample from the internal sensor solution. This solution contained NADH and semicarbazide, so that no reagent had to be added to the sample. The arrangement was named an 'internal optical enzyme sensor'.

3.1.7 Sensors for Phenols and Amines

Phenols are important intermediates of coal processing; their concentration in wastewater is relevant for pollution control. Furthermore, various drugs such as adrenaline and noradrenaline belong to this class of substances.

For the determination of phenols and amines enzymes with low selectivity, e.g. laccase, horseradish peroxidase, tyrosinase, and polyphenol oxidase, as well as specific enzymes, e.g. phenol hydroxylase and catechol-1,2-oxygenase, can be used in biosensors.

Laccase (EC 1.10.3.2) from *Polyporus versicolor* has a molecular weight of 60 000. The enzyme consists of two identical subunits, each containing two Cu^{2+} ions. It catalyzes the oxidation of a number of diphenols and diamines such as hydroquinone, noradrenaline, and p-phenylenediamine, by molecular oxygen. Wasa et al. (1984b) immobilized laccase by glutaraldehyde coupling to amino groups of a glassy carbon electrode. For detection of the reaction products, such as benzoquinone, the electrode was polarized to -100 mV vs SCE. The response time for hydroquinone was only 5 s and the detection limit was as low as $0.2 \mu\text{mol/l}$. This high sensitivity is a result of enzymatic-electrochemical analyte recycling during which the substrate is regenerated at the electrode (Fig. 60). The amplification factor was between 10 and 20. This type of substrate recycling has also been studied by Scheller et al. (1987b). When the electrode was polarized to -100 mV for hydroquinone indication, a usual linear concentration dependence was observed (Fig. 61). The slope, i.e. sensitivity, was 5 times higher than that obtained

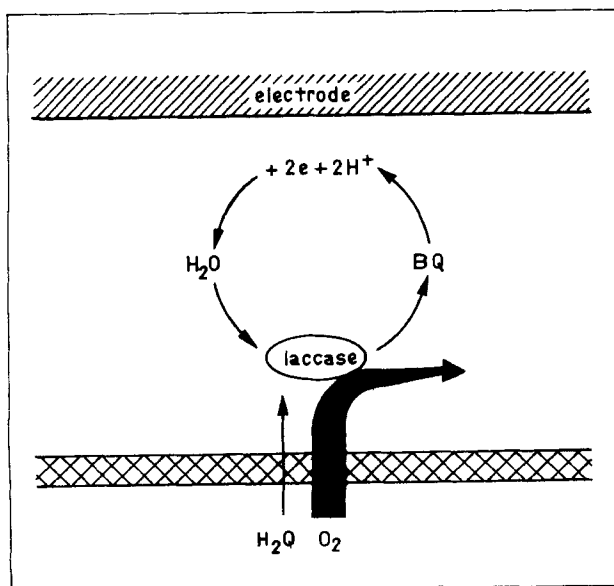


Fig. 60. Signal amplification by electrochemical regeneration of the laccase substrate, hydroquinone (H_2Q). BQ: benzoquinone.

with electrochemical substrate detection at +100 mV in the presence of the laccase inhibitor, azide. Evidently, the amplification mechanism described by Wasa et al. (1984) was active.

A laccase electrode for the determination of the lignin content of wood has been described by Malovik et al. (1983). The samples were extracted with organic solvent and the extracts directly injected into the measuring cell.

Laccase also catalyzes the O_2 -dependent oxidation of ascorbic acid, ferrocyanide, iodide, and uric acid. These reactions have been utilized to eliminate electrochemical interferences in amperometric hydrogen peroxide detection at membrane-covered enzyme electrodes (Wollenberger et al., 1986). The capacity of the laccase membrane to oxidize ferrocyanide has been characterized by anodic oxidation of ferrocyanide at +0.4 V (Fig. 62). When a fresh enzyme membrane is used, a current signal appears only at substrate concentrations above 5 mmol/l; the current increases linearly with increasing concentration. This threshold concentration decreases with increasing membrane age until the remaining enzyme activity is too low for complete substrate oxidation.

The flavoprotein phenol-2-hydroxylase (EC 1.14.13.7) and the o-phenol-splitting catechol-1,2-oxygenase (EC 1.13.1.1) are prepared from

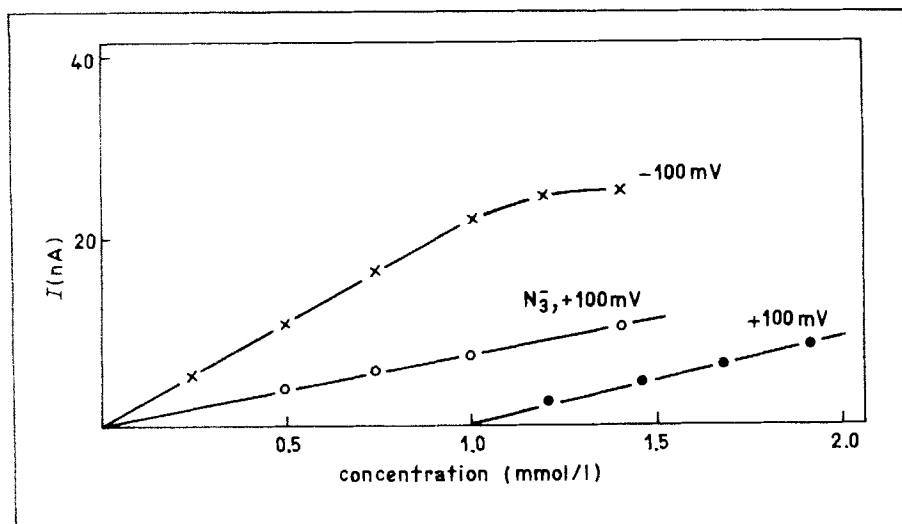


Fig. 61. Concentration dependence of the current of hydroquinone (H_2Q) conversion at a laccase electrode. At +100 mV H_2Q not converted enzymatically is directly oxidized at the anode whereas at -100 mV only the product of the enzymatic reaction is indicated.

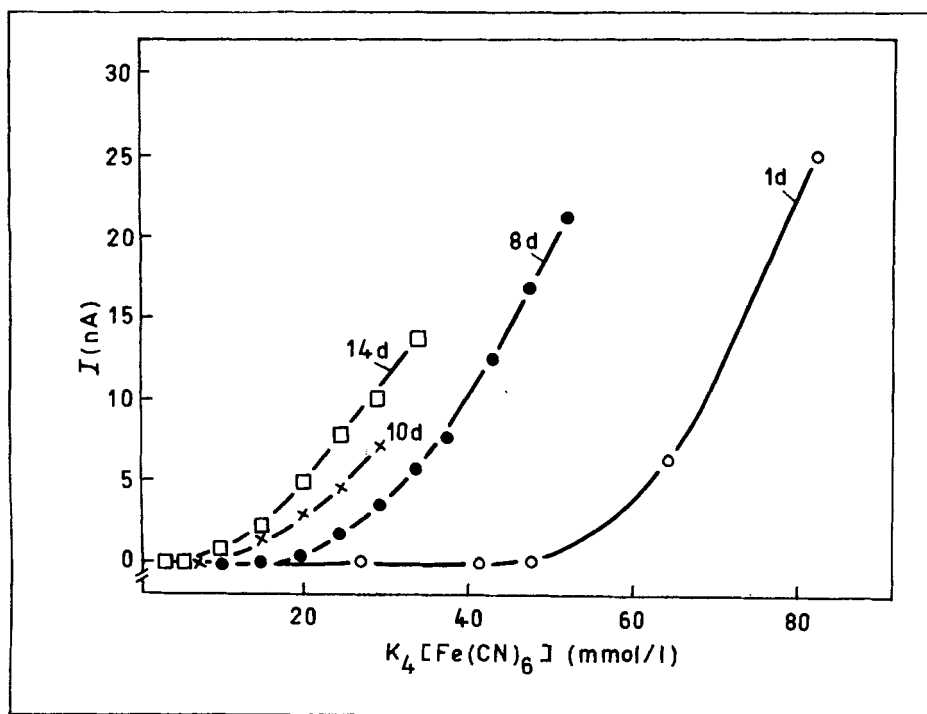
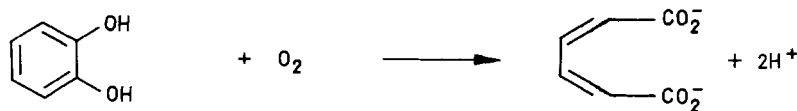
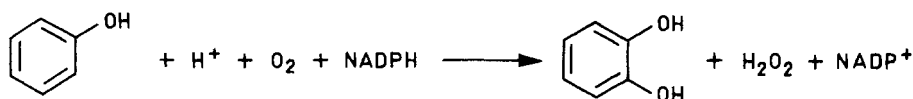


Fig. 62. Influence of the age of a laccase membrane on its capacity to oxidize ferrocyanide.

phenol-induced *Trichosporon cutaneum*. Both enzymes start the degradation of phenols in microorganisms (Neujahr, 1982):



Phenol-2-hydroxylase also oxidizes NADPH with the formation of H_2O_2 . Various monosubstituted phenols are also oxidized, but at a lower rate than phenol itself. Therefore the sensitivity of phenol hydroxylase enzyme electrodes based on O_2 indication as described by Kjellén and Neujahr (1980) is different for substituted and unsubstituted phenols. Consequently, in mixtures of phenols the oxygen consumption does not reflect the real phenol concentration.

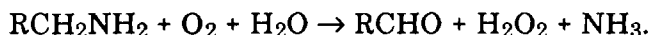
Specific catechol determination is demanded in the photographic, paint, and pharmaceutical industries. Neujahr (1980) combined immobilized catechol-1,2-oxygenase with an oxygen electrode and employed the sensor for catechol measurement. The sensor was selective for catechol and 3- and 4-methylcatechol. Phenol, resorcinol, cresol, vaniline, and dihydroxyphenylalanine (DOPA) did not interfere.

Phenols have also been determined by using the copper enzyme tyrosinase (EC 1.14.18.1). This enzyme oxidizes phenols and catechols to polymeric products with consumption of O_2 . Macholán (1979) immobilized tyrosinase by glutaraldehyde crosslinking with BSA to a nylon net and attached the resulting membrane to the tip of an oxygen probe. The sensor measured 4-chlorophenol and xylol as sensitively as phenol and hydroquinone, and pyrogallol with half the sensitivity. The low selectivity and the different sensitivity for different substances restricts the applicability of the sensor for substrate determination. On the other hand, the sensor is very suitable for enzyme activity measurement, since in this case an appropriate substrate can be used. The sensor was therefore applied to the measurement of the activity of β -glucosidase and alkaline phosphatase by following the formation rate of phenol from the appropriate phenol derivatives. For alkaline phosphatase phenolphosphate was used instead of the usual substrates, 2- or 4-nitrophenylphosphate, because nitrophenol is not oxidized by tyrosinase.

Tyrosinase also oxidizes p-cresol to 4-methyl-1,2-benzoquinone. In contrast to what happens in aqueous medium, nonpolymeric products are formed in an organic solvent. The quinone can be cathodically reduced at -275 mV vs SCE. This electrode reaction has been utilized in a biocatalytic electrode for the determination of phenols in chloroform (Hall et al., 1988). The enzyme was immobilized by allowing a tyrosinase solution to soak into the pores of a nylon membrane (Hybond N-nylon, Amersham International plc, UK). The enzyme-loaded membrane was then folded around a graphite foil electrode, which had been previously soaked in a solution of tetrabutylammonium toluene-4-sulfonate in chloroform. In chloroform saturated with 50 mmol/l aqueous phosphate buffer this enzyme electrode responded reproducibly to p- and m- cresol (not to o-cresol), phenol, and 4-chlorophenol in the concentration range 10–100 $\mu\text{mol/l}$. Operation of the sensor offers several advantages. By partitioning of an analyte of very low concentration from a large aqueous volume into a small organic volume the sensitivity could be dramatically increased. In this way the detection of phenolic compounds in wastewater could be quantified by the 'nonaqueous' enzyme electrode.

Horseradish peroxidase (HRP, EC 1.11.1.7) catalyzes the H_2O_2 -dependent oxidation of phenols and amines forming colored polymeric products via radical intermediates. This reaction has been used to detect phenol, bilirubin and aminopyrine (Renneberg et al., 1982). The hydrogen peroxide required was either injected into the measuring cell or generated in the enzyme membrane itself. For the latter reaction, GOD was coimmobilized with HRP.

Amines are formed in meat during storage. The monoamine content is therefore a measure of the meat freshness. Karube et al. (1980b) assembled a sensor for the determination of meat freshness based on monoamine oxidase (EC 1.4.3.4) catalyzing the reaction:



The enzyme was crosslinked by glutaraldehyde to collagen fibril and fixed to an O_2 probe. Maximal sensitivity was found with hexylamine and some other aliphatic monoamines. Tyramine and histamine were indicated with 30% sensitivity.

For the determination of biogenic amines, Toul and Macholán (1975) constructed an enzyme electrode containing immobilized diamine oxidase. The sensor was able to detect polyamines, putrescine, cadaverine, and histamine. In contrast, a plant polyamine oxidase (EC 1.5.3.3) electrode is highly specific for spermidine and spermine (Macholán and Jilkova, 1983). Ceruloplasmin (EC 1.16.3.1), a copper-containing glycoprotein, has been used in an enzyme sensor for aromatic amines and phenol (Macholán and Jilek, 1984). The sensor was more sensitive to ortho-substituted derivatives than to the corresponding para-compounds and did not react with m-diphenols and m-diamines.

L-carnitine was determined with an enzyme electrode composed of carnitine dehydrogenase and diaphorase in a chamber placed between a platinum disk and a semipermeable membrane (Comtat et al., 1988). In the presence of 20 mmol/l NAD^+ the oxidative current of liberated ferrocyanide was proportional to the concentration of carnitine between 0.1 and 10 mmol/l.

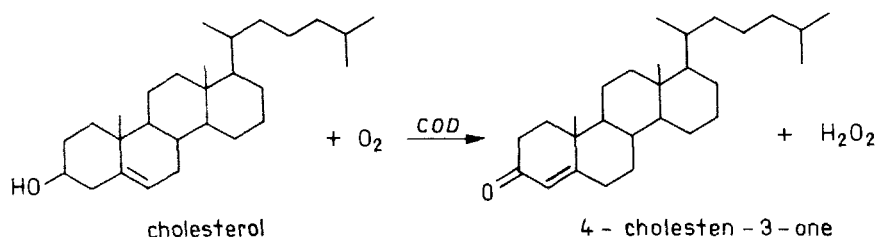
Aromatic amines are activators of horseradish peroxidase. Kulys and Vidziunaite (1983) adsorbed HRP together with GOD on a carbon electrode and crosslinked the enzymes with glutaraldehyde to assemble a sensor for aromatic amines. H_2O_2 is produced in the presence of glucose, acting as cosubstrate in the HRP-catalyzed oxidation of ferrocyanide. The ferricyanide formed was reduced back to ferrocyanide at an electrode potential of +10 mV vs SCE, the current being limited by the

reaction rate of HRP. Addition of aromatic amines increased the reaction rate and thus the steady current. The current increase was linearly dependent on activator concentration in the range 0.1–10 $\mu\text{mol/l}$. The maximum current increase was 600%. The effect of activation was much stronger when a solution of HRP was used; it may be concluded that diffusion plays a dominant role in the enzyme electrode function.

3.1.8 Cholesterol Sensors

Cholesterol is the most abundant steroid in man, occurring both as free cholesterol (about 30%) and esterified with fatty acids (about 70%). The assay of cholesterol is of great importance in the diagnosis of disturbances in lipid metabolism. The normal concentration range in blood serum is 3.1–6.7 mmol/l (Strassner, 1980). Cholesterol determination is also of importance in fermentation control and the pharmaceutical industry.

The basis for all enzymatic cholesterol assays is the hydrolysis of cholesterol esters by cholesterol esterase (CEH, EC 3.1.1.13) to free cholesterol and fatty acids and the oxidation of free cholesterol to cholestenone by cholesterol oxidase (COD, EC 1.1.3.6) with concomitant oxygen consumption and hydrogen peroxide formation:



In the latter, the stereospecific oxidation of the 3-hydroxyl group is rate-limiting because the subsequent isomerization at the Δ^5 position proceeds very rapidly. The reaction product, 4-cholesten-3-one, inhibits competitively ($K_i = 0.13 \text{ mmol/l}$).

Various COD enzymes have been isolated from microbes. Whereas extracellular COD has been shown to be an FAD enzyme, no prosthetic group could be identified in intracellular COD, e.g., from *Nocardia* sp. (Smith and Brooks, 1976). The molecular weight of COD is 35 000. The enzyme exhibits a broad pH optimum with maximum activity in 0.5 mol/l

phosphate buffer, pH 7.0 (Noma and Nakayama, 1976). The substrate is oxidized by a ping-pong mechanism with a K_M of 0.02 mmol/l (Richmond, 1973). The activation energy of the reaction amounts to 30.7 kJ/mol (Wollenberger, 1984). Although only the 3β -hydroxyl group of the steroid is oxidized, substituents at other positions may affect the reaction kinetics (Wortberg, 1975; Brooks and Smith, 1975; Richmond, 1973). Owing to the low solubility of the substrate in water, detergents have to be added to the reaction mixture. A solution containing 0.15% Triton X-100 has been established as optimal (Wollenberger, 1984).

For analytical purposes cholesterol oxidase has been immobilized on various carriers (Table 6). Electrochemical, optical, and calorimetric indication have been used as detection methods. Combination of a thermistor-coupled flow-through system with immobilized COD permitted the measurement of 0.03–0.15 mmol/l cholesterol (Mattiasson et al., 1976). Ögren et al. (1980) described an immobilized COD reactor for the analysis of steroid fractions obtained by high pressure liquid chromatography. The UV absorption at 240 nm of enzymatically formed cholestenone was used as the measuring signal. Linearity was found between 10 and 80 μ mol/l.

TABLE 6

Analytical Application of Immobilized Cholesterol Oxidase

Carrier	Product detection	References
Collagen	electrode	Satoh et al. (1977); Clark (1977) ¹
	reactor electrode	Coulet and Blum (1983)
Alkylamine glass	thermistor	Mattiasson et al. (1976)
	reactor electrode	Huang et al. (1977) ¹
	spectrophotometer	Tabata et al. (1981) ¹
Corning glass	spectrophotometer	Ögren et al. (1980)
Quartz tubes	lumometer	Rigin (1978)
Sepharose 4B	spectrophotometer	Mindner et al. (1978)
Cellulose	lumometer	Kobayashi et al. (1981)
Sepharose L-4B	reactor electrode	Karube et al. (1982b) ¹
Nylon	electrode	Mascini et al. (1983)
Glutaraldehyde	electrode	Nakamura et al. (1980)
Spheron ²	electrode	Wollenberger et al. (1983) ¹
Polyamide-6	electrode	Wollenberger (1984)

¹coimmobilization of COD and CEH

²2-hydroxyethyl methacrylate gel

For application in enzyme electrodes COD has been mainly immobilized by surface fixation (Table 7). As early as 1977, Clark had patented the polarographic analysis of free and esterified cholesterol by means of free as well as immobilized COD and CEH with anodic H_2O_2 indication. Cholesterol in food and serum samples has been determined by using COD bound to a collagen membrane via glutaraldehyde and coupled to a Pt electrode (Clark, 1978). A similar probe has been devised by Bertrand et al. (1979). In this sensor the enzyme membrane was not protected by a semipermeable membrane. Interferences were compensated for by difference measurements between an enzyme sensor and an enzyme-free membrane electrode. The lower detection limit was 0.05

TABLE 7

Biospecific Electrodes for Cholesterol

COD carrier	Detected substance	Range (mmol/l)	Response time (min)	Measuring time (min)	CV (%)	Life-time	References
Collagen	O ₂	0-0.2	5				Satoh et al. (1977)
Collagen	H ₂ O ₂	0.78-7.8	6-8		1.3		Clark et al. (1978)
Collagen	H ₂ O ₂	0.02-20	3-5		19.2	several months	Bertrand et al. (1979)
			0.75 ¹			150 det.	
Poly-amide-6	H ₂ O ₂	0.26-5.2		7	5	8 days	Wollenberger (1984)
				3 ¹	6 ¹	120 det.	
Spheron ²	H ₂ O ₂	0.4-12	2	4	2.7	10 days,	
			0.5 ¹		4.5 ¹	120 det.	Wollenberger et al. (1983)
Nylon	O ₂	0.01-0.13		4	6	20 days	Mascini et al. (1983)

¹kinetic method

²2-hydroxyethyl methacrylate

mmol/l. The reproducibility of serum measurement was poor, the CV being 19.2% (1.46 ± 0.28 mmol/l).

Wollenberger et al. (1983) described a sensor for free cholesterol containing COD bound covalently to spherical particles (Spheron). The sensor was capable of measuring 0.4–12 mmol/l cholesterol with good reproducibility and a response time in the rate regime of only 30 s (Fig. 63). The K_M value of the enzyme was increased by immobilization to 0.17 mmol/l. Investigation of the dependence of sensor response on stirring speed, temperature, and enzyme loading, as well as a linear Lineweaver–Burk plot and the fixation of COD solely on the carrier surface indicated kinetic control of the sensor.

Cholesterol electrodes based on the registration of oxygen consumption have also been described. The enzyme was immobilized to collagen membranes by crosslinking with BSA and glutaraldehyde (Sato et al., 1977) or bound to nylon (Mascini et al., 1983) and fixed in front of the O_2 probe. The sensor developed by Sato et al. has been applied to serum samples.

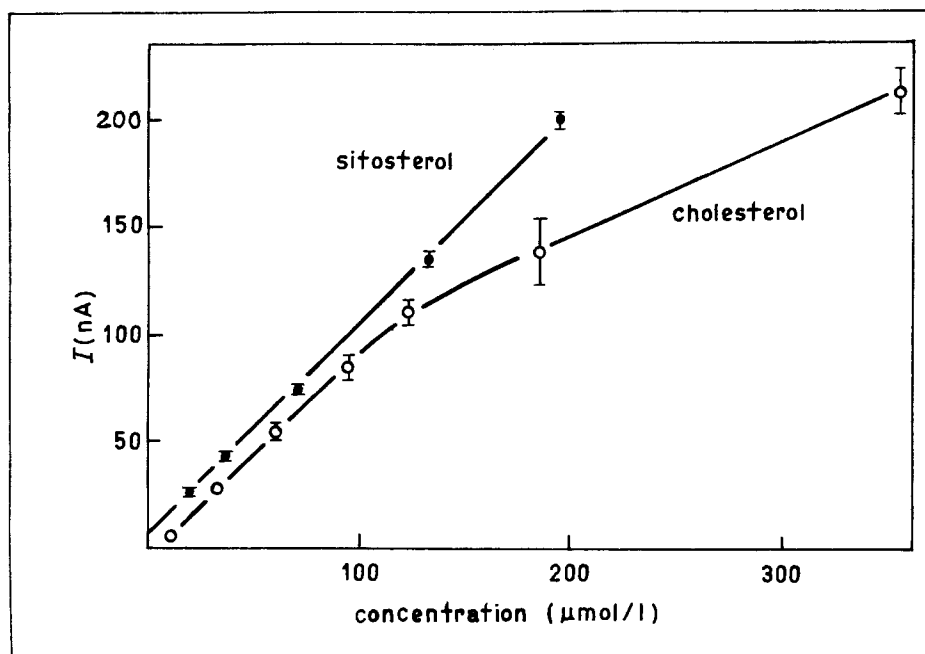


Fig. 63. Dependences of the hydrogen peroxide oxidation current of a cholesterol oxidase sensor on the concentration of cholesterol and sitosterol.

Wollenberger (1984) employed an immobilized COD electrode for the determination of sitosterol (Fig. 63). The decrease of the cholesterol signal by addition of cholestenone was used to measure this competitive inhibitor (Fig. 64).

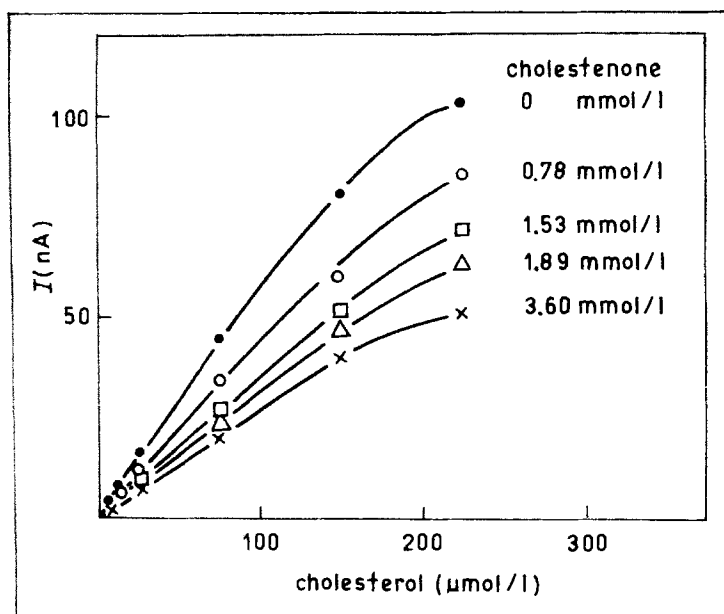


Fig. 64. Effect of the cholesterol oxidase inhibitor, cholestenone, on the measuring signal of a cholesterol oxidase sensor.

3.1.9 Determination of Bile Acids

The degradation of cholesterol leads to the production of bile acids which are structurally closely related to various steroid hormones. β -Hydroxysteroid dehydrogenase (EC 1.1.1.51) catalyzes the NAD^+ -dependent oxidation of 3β -, 17β - and some 16β -hydroxysteroids to the respective ketosteroids. The enzyme has been adsorbed on a carbon electrode modified by $\text{NMP}^+\text{TCNQ}^-$ and the NADH liberated in the reaction oxidized anodically (Albery et al., 1987a). Campanella et al. (1984) employed an enzyme sequence electrode composed of NAD^+ -dependent steroid dehydrogenase and horseradish peroxidase for assay of 7α -hydroxysteroids.

3.1.10 Determination of Glycolate, Glyoxylate and Hydroxybutyric Acid

Glycolic acid is an intermediate product of photosynthesis. Algae and cyanobacteria excrete glycolic acid during fermentation.

For determination of glycolate, glycolate oxidase (EC 1.1.3.1) has been entrapped by a dialysis membrane in front of a carbon electrode modified by 1,1'-dimethylferrocene (Dicks et al., 1986). With a response time of 2 min the oxidation current was proportional to glycolate concentration up to 5 mmol/l.

Depending on the redox state of the cofactor, in the presence of glyoxylate lactate dehydrogenase catalyzes either the reduction of glyoxylate to glycolate or the oxidation of glyoxylate to oxalic acid. The detection of the latter has been used in a bienzyme electrode containing LDH and oxalate oxidase (Schubert et al., 1990). In the presence of 2 mmol/l NAD^+ the current was linearly related to the concentration of glyoxylate up to 1 mmol/l. With a thermistor unit incorporating an LDH reactor a linear dependence of the heat of reaction on the concentration of both glyoxylate and glycolate was established (Danielsson et al., 1989).

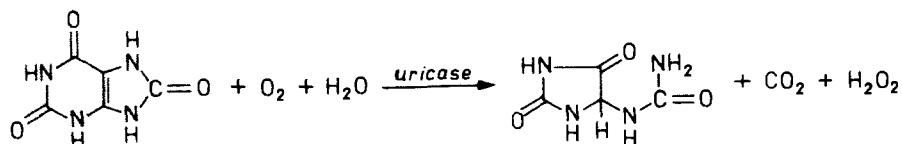
Similarly, the conversion of a homologous substrate has been demonstrated by using lactate oxidase. 2-Hydroxybutyric acid was measured with a lactate oxidase electrode with a sensitivity comparable with that for the 'normal' substrate, lactate.

3-Hydroxybutyrate, an important metabolite of glucose degradation, has been determined by using 3-hydroxybutyrate dehydrogenase (EC 1.1.1.30) (Palleschi et al., 1988). The enzyme was adsorbed on a porous carbon material and the formation of NADH was indicated at +0.3 V vs SCE. The sensor exhibited a linear calibration graph between 5 and 100 $\mu\text{mol/l}$ and was thus useful for assaying hydroxybutyrate in the physiological concentration range (50 $\mu\text{mol/l}$).

3.1.11 Determination of Uric Acid

Uric acid is the end product of purine metabolism in man. Consequently, abnormal levels of uric acid serve to indicate disorders of metabolism of purines or nucleic acids. Serum levels of uric acid range from 200 to 420 $\mu\text{mol/l}$. In males 15 to 20% and 3 to 4% of females suffer from hyperuricaemia.

The enzyme used for the assay of uric acid is uricase (urate oxidase, EC 1.7.3.3) which catalyzes the oxidation of uric acid to allantoin, CO_2 , and H_2O_2 :



The molecular weight of uricase is 100 000. The K_M for uric acid and the pH optimum vary over a wide range depending on the source of the enzyme.

According to the equation of the reaction, amperometric oxygen or hydrogen peroxide electrodes can be used to follow uric acid conversion. Potentiometric electrodes have seldom been employed because of their slow response (Kawashima and Rechnitz, 1976; Kawashima et al., 1980).

The operational parameters of amperometric uricase sensors are listed in Table 8. The first uric acid electrode was reported by Nanjo and Guilbault (1974a), who combined uricase crosslinked together with BSA by glutaraldehyde with an oxygen sensor. A similar enzyme preparation was directly fixed to a dialysis membrane by Kulys et al. (1985). Jänchen et al. (1983) entrapped uricase in gelatin and treated the membrane with a Cr^{3+} salt. Tsuchida and Yoda (1982) fixed the enzyme by chitosan in the pores of an asymmetric hydrogen peroxide selective membrane. A sensor bearing uricase immobilized on an asymmetric membrane is also being applied in the 'UA 300A' uric acid analyzer of Fuji Electric (Japan) (Yoshino and Osawa, 1980).

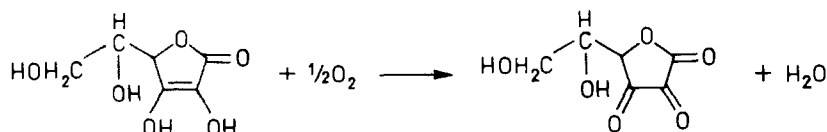
In view of the low physiological uric acid concentrations, in the assay of uric acid electrochemical interferences by other anodically oxidizable substances become particularly troublesome. Kulys et al. (1983) co-immobilized HRP with uricase in order to eliminate these interferences. This approach permits uric acid measurement to be performed at a potential of 0 V vs SCE. However, the autoxidation of ferrocyanide used as HRP substrate is a serious drawback of this method.

3.1.12 Determination of Ascorbic Acid (Vitamin C)

Ascorbic acid is oxidized with the consumption of oxygen in the reaction of ascorbic acid oxidase (EC 1.10.3.3):

TABLE 8
Uricase Electrodes

Detected substance	Sample volume (μl)	Linear up to (mmol/l)	Stability	CV (%)	Correlation with optical methods $y = ax + b; r$			References
					a	b (mmol/l)	r	
O ₂	500	0.5	100 days, 70% residual activity	4	0.96	0.049	1.02	Nanjo and Guilbault (1974a)
[Fe(CN) ₆] ³⁻ via HRP)	10	0.035	40 days, 50% residual activity		0.97	0.357	1.0	Kulys et al. (1983)
O ₂	100	1.2	7 days	3.2–4.8				Jänchen et al. (1983)
H ₂ O ₂	100	1.2		1.8–2.0	0.943	0.0198	0.9948	
H ₂ O ₂	20	0.6	500 samples	0.5–2.7	1.10	$2.44 \cdot 10^{-3}$	0.974	Yoshino and Osawa (1980)
H ₂ O ₂	25	3.0	17 days (1000 samples)	0.6–2.2	0.977	$3 \cdot 10^{-3}$	0.985	Tsuchida and Yoda (1982)



The enzyme consists of two identical subunits, each having a molecular weight of 72 000. The K_M values are 1.09 mmol/l for ascorbic acid and 0.59 mmol/l for oxygen. The pH optimum is at pH 6.0.

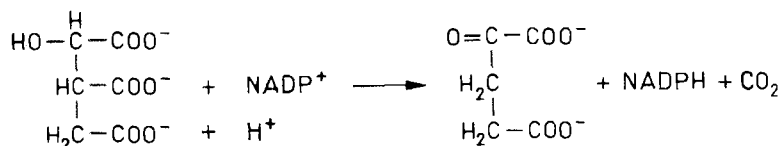
Posadaka and Macholán (1979) and Matsumoto et al. (1981) combined glutaraldehyde-crosslinked ascorbate oxidase with O_2 electrodes. The obtained sensors were applied to the determination of vitamin C in food samples. The only compounds interfering were L-iso-ascorbic acid and triosereductone.

Wasa et al. (1984a) developed a chemically modified enzyme electrode for ascorbic acid. The enzyme was attached to impregnated highly porous carbon together with BSA by glutaraldehyde. In intensively stirred solution the response time of the sensor was only 3 s. The sensitivity dropped to 80% within 3 months.

Nagy et al. (1982) employed an ascorbate oxidase membrane to eliminate the oxidation current caused by ascorbic acid during the microelectrochemical measurement of catecholamines in brain. The membrane was attached to a carbon microelectrode and was able to completely oxidize the penetrating ascorbic acid to electrochemically inert dehydroascorbic acid whereas the catecholamines could diffuse to the electrode. The sensor was called an 'eliminator electrode'.

3.1.13 Determination of D-Isocitrate

D-isocitrate is an intermediate product of the citrate cycle. It is formed as a by-product of the fermentative production of citric acid. The enzymatic assay of isocitrate is based on the oxidative decarboxylation to α -ketoglutarate in the presence of isocitrate dehydrogenase (EC 1.1.1.42):

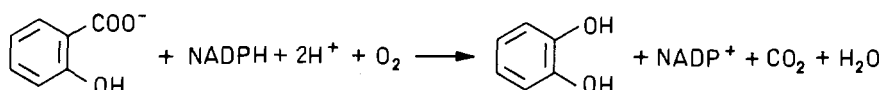


The reaction has been coupled to the electrochemical detection of the liberated NADPH at a chemically modified carbon electrode (Nakamura

et al., 1980). Schubert et al. (1985b) developed an assay method based on an isocitrate dehydrogenase–horseradish peroxidase sequence electrode. The NADPH formed in the dehydrogenase reaction is oxidized under O_2 consumption by HRP. $MgCl_2$ was used as the activator of isocitrate dehydrogenase and Mn^{2+} ions served as co-catalyst for the NADPH oxidation. Ten isocitrate determinations per hour could be performed with the sensor. The detection limit was 0.2 mmol/l.

3.1.14 Salicylate Sensors

Acetylsalicylic acid is widely used as an analgesic and anti-inflammatory agent. In the body it is hydrolyzed to salicylate. The assay of salicylate by enzyme electrodes is based on the reaction of salicylate hydroxylase (EC 1.14.13.1):



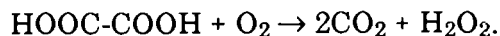
Fonong and Rechnitz (1984a) entrapped the enzyme physically with a dialysis membrane at the sensing tip of a carbon dioxide selective electrode. The sensor was suitable for salicylate concentrations in the range 0.04–2.2 mmol/l.

Rahni et al. (1986b) combined immobilized salicylate hydroxylase with an oxygen electrode. With a linear measuring range of 0.01–0.7 mmol/l, salicylate concentrations in serum could be measured without preconcentration. The sensitivity of the enzyme electrode for aspirin and gentisate was 20 times lower than for salicylate.

3.1.15 Determination of Oxalate and Oxaloacetate

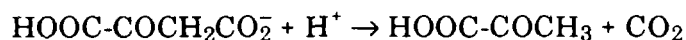
The determination of oxalate in urine is required for diagnosis of renal calculus and hyperoxaluria. For enzymatic oxalate determination, oxalate decarboxylase (EC 4.1.1.2) has been employed in enzyme thermistors (Danielsson et al., 1981), enzyme reactors (Lindberg, 1983), and potentiometric enzyme electrodes (Kobos and Ramsey, 1980).

Alternatively, oxalate oxidase (EC 1.2.3.4) can be used, which catalyzes the following reaction:



It is evident from the equation that potentiometric CO₂ electrodes as well as amperometric O₂ or H₂O₂ electrodes can be used as transducers. Both potentiometric and amperometric sensors have been covered by a layer of oxalate oxidase protected by a dialysis membrane (Bradley and Rechnitz, 1986; Rahni et al., 1986a). The sensors had a pH optimum at pH 3.5–4. Diffusion control was reached at 1 U oxalate oxidase per electrode. Oxalate determination was not affected by ascorbic acid or amino acids. The hydrogen peroxide-detecting sensor (Rahni et al., 1986a) has been used to measure oxalate in urine diluted 1:40.

Enzyme sensors for oxaloacetate are based on immobilized oxaloacetate decarboxylase (EC 4.1.1.3) and have been applied to the determination of aspartate aminotransferase (see Section 3.2). Ho and Rechnitz (1987) suggested combining partially quarternized polyethyleneimine catalyzing the reaction:

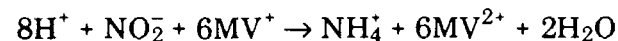
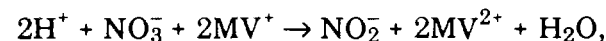


with a carbon dioxide electrode. This oxaloacetate sensor can be regarded as the first 'synzyme electrode'.

3.1.16 Determination of Nitrite and Nitrate

Nitrite ion has been shown to be toxic in the human body and animals, since diazotization of amines present in the body can lead to carcinogenic N-nitroso compounds. Furthermore, nitrite oxidizes hemoglobin to methemoglobin which is incapable of binding oxygen. In contrast, nitrate itself is not toxic but is easily reduced to nitrite by microorganisms.

Enzymatic assay of nitrite and nitrate is based on the following reactions catalyzed by nitrate reductase (EC 1.9.6.1) and nitrite reductase (EC 1.6.6.4):



where MV²⁺ represents oxidized methylviologen.

Nitrate is reduced to ammonia via intermediate nitrite with the participation of reduced methylviologen as electron donor.

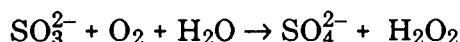
Kiang et al. (1978) developed an enzyme reactor incorporating the immobilized reductases. The eluate from the reactor was mixed with NaOH to expel the liberated NH₃ gas which was subsequently detected

by using an air gap electrode. The use of two enzyme columns, one containing nitrite reductase and the other nitrate reductase, enabled the measurement of nitrite and nitrate separately.

Direct coupling of the enzymes with the ammonia electrode is not possible, since the sensitivity of the electrode is too low at the optimum pH of the reductases (7.4–7.8).

3.1.17 Sulfite Sensors

Sulfite is used in large amounts in paper production and as a reductant in various chemical reactions. Fonong (1986a) immobilized sulfite oxidase (EC 1.8.3.1) covalently on a collagen membrane which was attached to a hydrogen peroxide-indicating Pt electrode. H_2O_2 liberated in the enzymatic reaction:



was detected at +0.7 V. The current was linearly related to sulfite concentration over the range of 1 to 150 $\mu\text{mol/l}$. The lower detection limit was 0.2 $\mu\text{mol/l}$.

The application of immobilized sulfite oxidase in the Multipurpose Bioanalyzer (Provesta, USA) has been described by Smith (1987). 1.8 U of the enzyme was entrapped in a gel at the active tip of an oxygen probe. The sensor is suitable for sulfite determination in food samples down to 100 ppm and has a lifetime of 84 days.

3.1.18 Determination of Carbon Monoxide

Carbon monoxide is highly toxic because it has a higher affinity for hemoglobin than does oxygen. CO sensors are therefore of utmost importance for combustion control, fire-alarm systems, and detection of hazardous concentrations of the gas in private and industrial facilities such as tunnels, coal mines, and underground garages. The sensors in current use, in which CO is anodically oxidized or adsorbed onto coated piezoelectrodes, are relatively nonspecific, since H_2S , N_2O , and NO_2 interfere.

Hill et al. (1981) proposed a CO sensor based on inhibition by the gas of cytochrome oxidase (EC 1.9.3.1) coupled to a modified platinum electrode via cytochrome c. This type of sensor has been further improved by Albery et al. (1987a).

Carbon monoxide oxidoreductase has been employed by Turner et al. (1984) in an attempt to construct a novel CO sensor. The enzyme, which was isolated from *Pseudomonas thermocarboxydovorans*, has a molecular weight of 270 000 and contains FAD, FeS, and molybdenum as redox active groups. It catalyzes the oxidation of CO to CO₂ using various electron acceptors, e.g. NMP⁺ or oxygen. The temperature optimum of the reaction is 80°C. The K_M value for CO is as low as 0.5 µmol/l; acetylene and cyanide inhibit the enzymatic reaction.

A biofuel cell containing the enzyme and NMP⁺ in the anodic half cell was suited for CO determination between 0.02 and 0.2 µmol/l. To achieve a sensor configuration, the authors embedded a conducting paste consisting of graphite powder, 1,1'-dimethylferrocene, and paraffin oil in the cavity of a platinum disk electrode. A solution of CO oxidoreductase was pipetted onto the paste surface and covered by a dialysis membrane (Fig. 65). The Ag/AgCl reference electrode was integrated into the sensor. The electrode was polarized to +160 mV and the anodic current used as the measuring signal. The sensor has been used for CO measurement in aqueous solution. The current change was completed within 15 s after sample injection and depended linearly on CO concentration up to 60 µmol/l. With a sensitivity decrease of 12%/h the operational stability was rather poor.

In principle, this sensor is also applicable to CO measurement in the gas phase for it was possible to keep the enzyme stable in a wet medium behind the gas-permeable membrane. In comparison with other biocatalytic gas-sensing devices, e.g. those for methane (Karube et al., 1982a) or NH₃ and NO₂ (Hikuma et al., 1980b) the sensor was more compact and its response was substantially faster. This enzyme electrode therefore represents a promising approach to novel gas sensors.

3.1.19 Electrochemical Sensor for Hydrogen Determination

The anodic and enzymatic oxidation of hydrogen in biofuel cells has been investigated to achieve efficient energy conversion. Hydrogenase (EC 1.98.1.1), a protein containing FeS-clusters, oxidizes H₂ with reduction of methylviologen. The reoxidation of the reduced mediator in deaerated solution gives a concentration-dependent oxidation current (Varfolomeev and Bachurin, 1984). This system has been adapted for analytical purposes by Boivin and Bourdillon (1987). The enzyme was attached covalently to the surface of a carbon electrode.

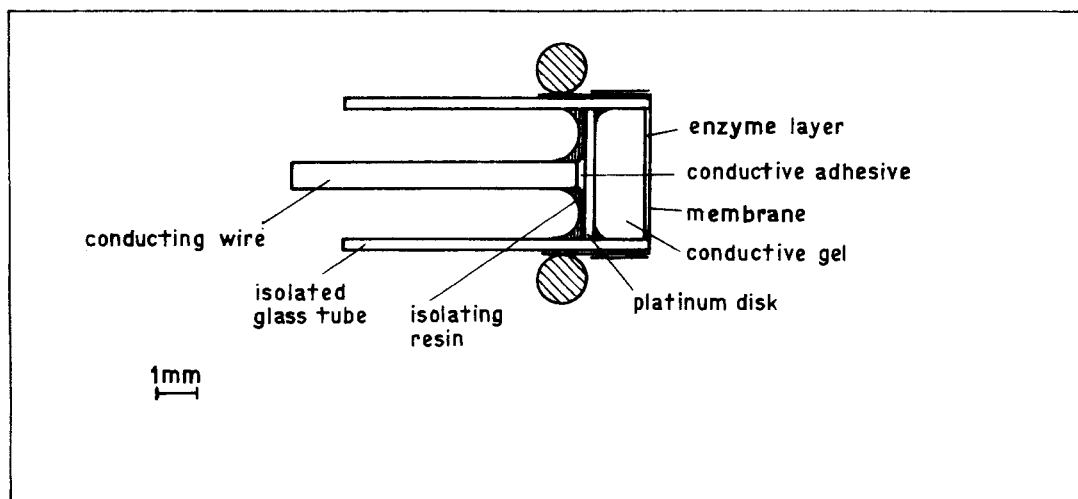
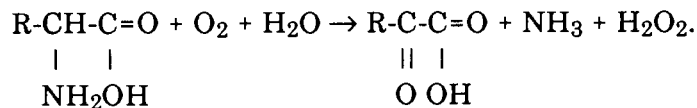


Fig. 65. Construction of an enzyme electrode for the determination of carbon monoxide gas. (Redrawn from Turner et al., 1984).

3.1.20 Sensors for Amino Acids

Amino acids constitute the second largest source of nonprotein nitrogen in serum, urea being major source. The determination of total serum amino acids can provide valuable clinical information. Single amino acids are measured to gain access to particular enzyme activities: of transaminases and peptidases for instance. Amino acids are also important in the food industry and in biotechnology. Their concentration in food can be used as a measure of the nutritive value of the food.

Two enzymes with a broad substrate specificity have been utilized in biosensors for amino acids: L-amino acid oxidase (EC 1.4.3.2) and D-amino acid oxidase (EC 1.4.3.3). They catalyze the irreversible formation of the respective α -keto acids:

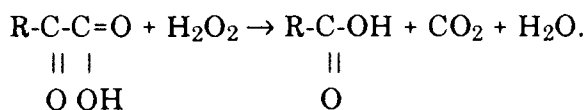


This reaction can be coupled to oxygen- and hydrogen peroxide-sensing electrodes (Guilbault and Lubrano, 1974) as well as to potentiometric pH, NH_3 , or NH_4^+ electrodes (Guilbault and Hrabankóva, 1971).

The following substrates can be determined with almost identical sensitivity by using D-amino acid oxidase in combination with an ammonium ion sensitive electrode: D-alanine, D-leucine, D-norleucine, D-methionine, and D-phenylalanine. L-amino acid oxidase sensors have been described for L-leucine, L-cysteine, L-methionine, L-tryptophan, and L-tyrosine (Guilbault and Hrabankóva, 1971), and L-histidine and L-arginine (Tran-Minh and Broun, 1975).

Yao and Wasa (1988a) assembled modified electrodes for amino acids by crosslinking L- or D-amino acid oxidase with glutaraldehyde on silanized platinum probes. The sensors were employed as detectors in high pressure liquid chromatography. Whereas the L-amino acid oxidase electrode responded to L-tyrosine, L-leucine, L-methionine, and L-phenylalanine in amounts as low as 2 pmoles, the D-amino acid electrode measured only D-methionine and D-tyrosine. The response time in steady state measurements was only 5–10 s.

Guilbault and Lubrano (1974) proposed an H_2O_2 -detecting L-amino acid oxidase sensor which exhibited negligible sensitivity to alanine and glycine. Cysteine was indicated by direct anodic oxidation at the electrode with a higher sensitivity than all other amino acids. Obviously no complete substrate conversion was achieved within the enzyme membrane. In another study, Nanjo and Guilbault (1974b) obtained a higher sensitivity by using an oxygen electrode instead of an H_2O_2 electrode. This finding was explained by the consumption of H_2O_2 during the nonenzymatic oxidation of α -ketoacids:



A major drawback of sensors using nonspecific amino acid oxidases is their differing sensitivity for different substrates, so that determination of the total amino acid concentration is not possible. Moreover, the sensitivity for a particular amino acid changes depending on the degree of kinetic limitation, i.e., it is also influenced by the duration of the sensor application (Scheller et al., 1989a).

Recently, selective oxidases for L-lysine (EC 1.4.3.-) (Romette et al., 1983) and L-glutamate (EC 1.4.3.11) have been employed in enzyme electrodes. Wollenberger et al. (1989) developed a sensor based on glutamate oxidase from *Streptomyces endus*, which permits selective measurement of glutamic acid between 1 $\mu\text{mol/l}$ and 1 mmol/l with a sample frequency of 120/h. In contrast, a sensor using an analogous

enzyme from *Streptomyces* sp. responded to glutamine with 3% and aspartate with 1% of the sensitivity for glutamate (Yamauchi et al., 1983). In both electrodes α -ketoglutarate competitively inhibited glutamate oxidase.

As early as 1975, Ahn et al. developed an enzyme electrode for glutamate composed of L-glutamate decarboxylase (EC 4.1.1.15) and a carbon dioxide sensor.

Glutamate dehydrogenase (EC 1.4.1.3) has been used for glutamate determination (Schubert et al., 1986b) as well as for quantitative removal of ammonia (Mascini et al., 1985a) (see Section 3.2).

Decarboxylases of phenylalanine, tyrosine, and lysine and ammonia lyases of histidine, glutamine, and asparagine are also highly selective. Guilbault et al. (1988) described a potentiometric enzyme sensor for the determination of the artificial sweetener aspartame (L-aspartyl-L-phenylalanine methylester) based on L-aspartase (EC 4.3.1.1). The ammonia liberated in the enzyme reaction created a slope of 30 mV/decade for the enzyme-covered ammonia sensitive electrode. The specificity of the sensor was excellent; however, the measuring time of 40 min per sample appears not to be acceptable. The measuring time has been decreased to about 20 min by coimmobilizing carboxypeptidase A with L-aspartase (Fatibello-Filho et al., 1988).

Histamine has been determined with an ion selective membrane electrode based on p-fluorophenyl borate (Katsu et al., 1986).

Toyota et al. (1985) developed a protein assay with use of a tyrosinase sensor. Tyrosine was measured after complete proteolysis of the sample by pepsin. The results agreed well with those obtained by the method of Lowry.

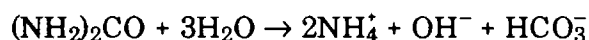
Fu et al. (1988) established a proteolytic enzyme-FET by immobilizing α -chymotrypsin on the gate of an ISFET. The signal is formed by the cleavage of N-benzoyl or N-acetyl esters of amino acids to the respective free acids. The response of these sensors was markedly affected by the pH and the concentration of the working buffer. The authors suggested that such sensors would be useful for protein assay in biological samples.

3.1.21 Urea Sensors

Urea is the most important end product of protein degradation in the body. The urea content of blood serum depends on protein catabolism and nutritive protein intake and is regulated by renal excretion. There-

fore the serum concentration of urea provides information on kidney function. Urea measurement is also relevant to the evaluation of extra-corporal detoxification procedures.

All enzymatic methods for the determination of urea are based on urea hydrolysis by urease (EC 3.5.1.5):



followed by assay of the liberated ammonia or carbon dioxide. A widely used method is the oxidative coupling of NH_3 with phenol to form indophenol (Berthelot reaction). Alternatively, NH_3 is measured by following the NADH consumption during enzymatic glutamate synthesis from ammonium ion and α -ketoglutarate as catalyzed by glutamate dehydrogenase.

Urease has a molecular weight of $590\,000 \pm 30\,000$ and consists of six identical subunits. Each subunit contains two Ni ions of different valency which are involved in substrate binding and conversion. The isoelectric point of the protein is at pH 5 and the temperature optimum of the catalysis at 60°C . The kinetic constants for urea hydrolysis have been determined to be $k_{+2} = 5870\text{ s}^{-1}$ and $K_M = 2.9\text{ mmol/l}$. Other amides, such as formamide and semicarbazide, react much more slowly than urea. The pH optimum of urease depends on the nature of the buffer used and, with the exception of acetate buffer, equals the $\text{p}K_s$ value of the buffer. The active center of urease contains an SH-group that is essential for the stability of the enzyme. Complexing agents, such as EDTA and reductants, are required for stabilization.

Most potentiometric biosensors are urea sensors. The first was developed by Guilbault and Montalvo (1969), who combined immobilized urease with a cation selective glass electrode. By the use of NH_4^+ sensitive electrodes and pH sensitive metal sensors the development route led to enzyme-FETs based on NH_3 -indicating Pd/Ir-MOS condensators or pH sensitive Si_3N_4 -coated FETs. In addition to these electrochemical urea sensors optical systems, test strips, and urease enzyme reactors have been proposed. Finally, some urea sensors are based on higher integrated biocatalytic systems such as microorganisms and tissue slices. Though prosecuted with nearly the same intensity as that of glucose sensors, these studies have not yet led to urea analyzers suitable for routine application.

3.1.21.1 Urease Reactors

A urease packed bed reactor combined with an ammonia electrode is being used in a blood urea nitrogen (BUN) analyzer marketed since 1976 by the Kimble Division of Owens-Illinois (USA). Urease is bound to aluminum oxide particles. The reaction is conducted at pH 7.5. In order to obtain a high sensitivity for the ammonia electrode, the product stream leaving the reactor is mixed with NaOH until a pH of 11 is attained (Watson and Keyes, 1976).

Gorton and Ögren (1981) introduced a reactor containing urease bound to porous glass in a flow injection analysis system. The product was detected by means of an ammonia electrode after alkalization of the measuring solution. A sample frequency of 60/h was obtained. No addition of alkali is necessary in a system using a urease reactor and a palladium-iridium-MOS condensator in an FIA device (Winquist et al., 1984). An enzyme thermistor containing immobilized urease also permits the direct determination of urea (Danielsson et al., 1976). Since the reaction enthalpy of urea hydrolysis is only -6.6 kJ/mol, the lower detection limit of the thermistor was not lower than 0.01 mmol/l. On the other hand, the measuring range extended up to 200 mmol/l.

Besides these conventional reactors with spherical immobilizates, urease has also been immobilized inside nylon tubing and pipette tips ('enzyme pipette', Sundaram and Jayonne, 1979), on nylon fibers ('enzyme brush', Raghavan et al., 1986), and on the surface of a magnetic stirrer (Guilbault and Starklov, 1975). The urease reaction was in each case carried out at optimal pH; after removal of the immobilized enzyme NH_3 was assayed electrochemically or photometrically according to Berthelot's method.

Seifert et al. (1986) coupled an integrated optical sensor bearing a miniature flow-through cell with a urease reactor (Fig. 66). To construct the sensor, a planar, light-conducting SiO_2 - TiO_2 structure was patterned by photolithography and etching in a grid relief on a Pyrex support. The sensor functioned as a differential refractometer indicating changes of the index of refraction at the sensor surface as well as the thickness of layers adsorbed on the grid region. Its applicability to urea measurement and immunoanalysis has been demonstrated.

3.1.21.2 Membrane Sensors

In contrast to reactor arrangements, the intimate physical contact of urease with the signal transducer in enzyme membrane sensors does not permit shifting the sample pH in favor of optimal transducer sensi-

tivity. Only a few urease sensors contain the enzyme in a separately prepared membrane; in most cases the active tip of the transducer is coated with a mixture of enzyme and carrier material. Sensors with urease covalently bound to the surface have not been described so far. Santhanam et al. (1977) adsorbed urease onto the surface of a mercury-covered thermistor. Upon addition of urea a temperature change was found, indicating that the enzyme retained its activity.

1. Potentiometric Electrodes

Most urea sensors are based on potentiometry. The selectivity of the urea sensor described by Guilbault and Montalvo (1969) (see above) has been improved by replacing the glass electrode by an NH_4^+ sensitive electrode based on nonactin (Guilbault and Nagy, 1973). The antibiotic was contained in a membrane of silicon rubber.

A very successful sensor suitable for urea measurement in blood has been developed for Hitachi (Japan) by Tokinaga et al. (1984). Two NH_4^+ sensitive electrodes containing nonactin in a PVC membrane were integrated in an FIA device in a differential circuit. One electrode was

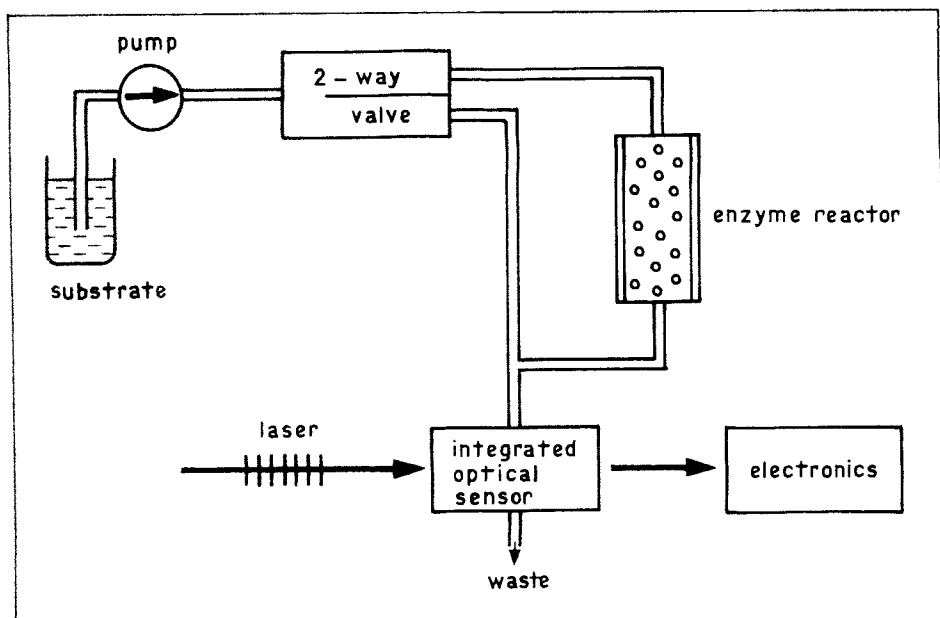


Fig. 66. Schematic of an optical biosensor for urea. The electronic part consists of a fiber optic sensor, a photomultiplier, and a lock-in amplifier.

covered by a polyester membrane (thickness, 0.025 mm) with immobilized urease. The lifetime of this membrane was determined to be 2 months. Using a sample volume of only 10 μl , 60 samples per hour could be analyzed.

Potentiometric gas sensors for the reaction products, NH_3 and CO_2 , have also been employed. Since these measurements are based on gas diffusion through a hydrophobic membrane, no direct disturbances by sample constituents occur. As early as 1969, Guilbault et al. coupled immobilized urease with a carbon dioxide sensor. Anfält et al. (1973) applied an ammonia gas sensitive electrode to urea assay. A major drawback of these sensors is their long response time which is due to the slow diffusion of the gases. Since it takes several additional minutes to reach a new baseline after each measurement, only a few samples can be processed per hour. Guilbault et al. (1985) therefore tried an NH_3 electrode, the internal buffer of which was exchanged after each measurement (double injection electrode). This approach led to a substantial decrease of the washing time.

Guilbault and Tarp (1974) succeeded in reducing the measuring time to 2–4 min per sample by replacing the gas sensitive membrane of NH_3 sensors by an air gap.

A further disadvantage of potentiometric gas sensors is the difference between the pH optima of the electrode and urease. Thus, NH_3 electrodes are operated at a pH around 8 while the optimal pH of the urease reaction is at pH 7.

Kobos et al. (1988) described the adsorption of urease on a fluorocarbon membrane for the construction of a urea sensor. The spontaneous adsorption was enhanced by a factor of 7 by perfluoroalkylation of the amino groups of the enzyme. The enzyme membrane was attached to an ammonia gas-sensing electrode. The urea sensor thus prepared exhibited a sensitivity of 50 mV per decade of urea concentration and a response time of 3 min. Only a small amount of enzyme could be adsorbed on the limited membrane surface, so the sensor was stable for only 7 days.

The pH increase caused by urea hydrolysis can also be indicated by using pH sensitive glass or metal oxide electrodes. Owing to the dissociation equilibria of NH_4^+ and HCO_3^- in the neutral range approximately 1 mole of OH^- ions is formed per mole of urea. Blaedel et al. (1972) showed that in diffusion controlled potentiometric enzyme electrodes, i.e. when the substrate is completely consumed within the enzyme membrane, the product concentration at the electrode surface depends

linearly on the substrate concentration. According to the Nernst equation, then, in the neutral range, a slope of the calibration graph of 59 mV per urea concentration decade is obtained.

To construct urea sensors based on pH electrodes urease has been immobilized by physical entrapment around the active sensor tip by a dialysis membrane (Nilsson et al., 1973) and by crosslinking with BSA and glutaraldehyde (Tran-Minh and Broun, 1975). For the latter, the sensor tip was dipped into the reaction mixture, allowed to dry in the air, and washed with glycine buffer to remove excess glutaraldehyde. The pH measured with this sensor was proportional to the logarithmic values of urea concentration in the range of 0.05 to 5 mmol/l.

Hamann et al. (1988) developed a urea sensor based on a pH glass electrode (Forschungsinstitut Meinsberg, GDR) and urease from soy beans with a specific activity of 2 U/mg. The enzyme was immobilized by dipping the wetted sensor tip first into crystalline urease and then into a solution of methylene chloride containing 21–25 mg/ml of cellulose triacetate. After evaporation of the solvent an amount of enzyme corresponding to 3 U remained at the sensor. The layer thickness and the diffusion coefficient of urea in the layer were determined to be 30–50 μm

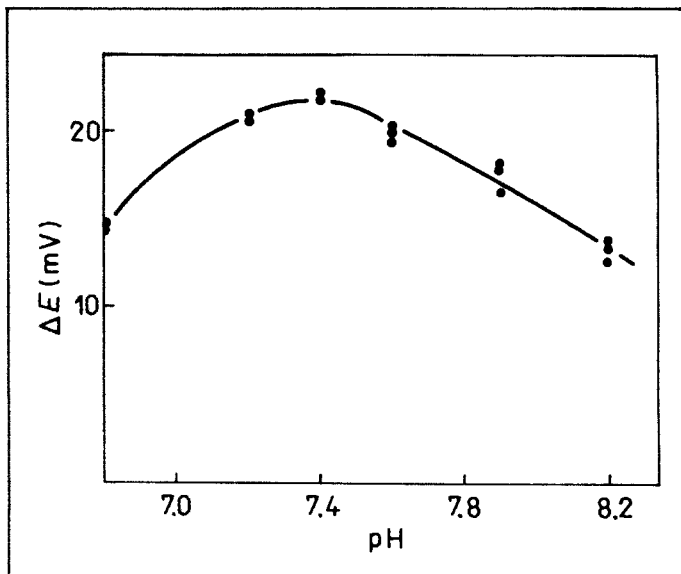


Fig. 67. Influence of sample pH on the measuring signal of a urease electrode 30 s after addition of 13.3 mmol/l urea to the measuring solution.

and $3 \cdot 10^{-10}$ m²/s, respectively, giving an enzyme loading factor, f_E , between 0.002 and 0.04. This extremely low value indicates that the sensor response was exclusively limited by the rate of the enzyme reaction. This was confirmed by the finding that the sensor contained an apparent enzyme activity of 2.4 U, i.e., almost all enzyme applied during immobilization remained active and was acting on the substrate. Furthermore, the apparent K_M value of 2.5 mmol/l as estimated from the initial rate of the potential change was not significantly different from that of the free enzyme. In these measurements a partition coefficient of 1.3 was taken into account.

The sensor exhibited optimal sensitivity at pH 7.4 (Fig. 67). In spite of the low enzyme loading, a functional stability of 4–6 weeks was assured by storing the sensor overnight in a cold solution containing EDTA and mercaptoethanol.

The mechanically unstable glass electrodes in urea sensors have been replaced by antimony (Joseph, 1984), iridium (Ianiello and Yacynych, 1983), and miniature palladium electrodes (Szuminsky et al., 1984). Urease was included in a PVC sheath or crosslinked by glutaraldehyde directly on the electrode surface. Inactivation by heavy metals was diminished by addition of EDTA.

The signals of those urea sensors that work without addition of alkali for ammonia liberation are severely affected by variations in buffer capacity and the pH of the sample. In addition to the pH change due to urea hydrolysis, the pH electrode of the sensor measures the pH change in the bulk phase generated by sample injection. This problem is analogous to that presented to oxygen-indicating glucose sensors by differences in the oxygen content of the background solution and the sample. In fact, an analogous principle has been employed to eliminate this effect, namely the implementation of difference measurement using a urease electrode and an electrode with no enzyme. Guilbault and Hrabankóva (1970) measured urea in blood and plasma by evaluating the difference between the stationary signals of two such sensors and obtained good agreement with a spectrophotometric reference method. The principle was further developed by Vadgama et al., (1982), who constructed a measuring device with two high-impedance input amplifiers tapping the difference signal directly. A considerable reduction in measuring time has been obtained by evaluation of the potential difference between the two pH electrodes as soon as 30 s after sample addition (Hamann et al., 1986).

Joseph et al. (1984) combined two antimony microelectrodes of 50 μ m

diameter to form a difference system without a reference electrode. One of the electrodes was covered with urease crosslinked with glutaraldehyde. In unstirred solution the electrode had a response time of only 1 min and the calibration curve exhibited a slope of 40–45 mV per decade of urea concentration. The useful lifetime was 3 months. In this arrangement the influence of sample pH is eliminated; no information is given on the effect of buffer capacity, though.

Kulys et al. (1986b) studied urea determination by difference measurement between two antimony electrodes covered with exchangeable membranes (Fig. 68). Urease was attached in the pores of a macroporous membrane (thickness, 10 μm , pore diameter, 0.1 μm) by glutaraldehyde. This layer was covered with a monoacetylcellulose membrane. The membrane for the auxiliary electrode was prepared analogously, but using BSA instead of urease. The assay of urea was carried out with a differential amplifier which simultaneously differentiated the time course of the potential difference between enzyme and auxiliary electrode (kinetic method). Thus, a response time of only 20 s was possible.

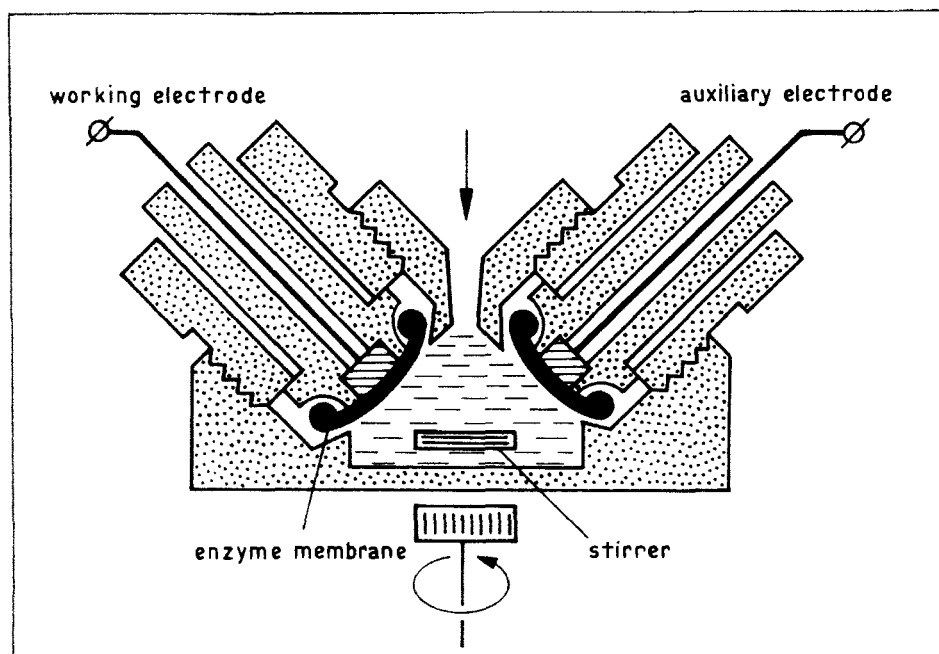


Fig. 68. Urea measuring system using two antimony electrodes. (Redrawn from Kulys et al., 1986b).

The calibration graph was *linear* between 0.2 and 2 mmol/l urea. Half of the initial sensitivity was lost after 16 days of operation. Despite the difference measurement the results obtained with blood samples correlated poorly with those determined by a reference method.

For small pH changes ($\Delta\text{pH} < 0.1$), constancy of the buffer capacity, β , of the measuring solution can be assumed. Hence, the following *linear* dependence of the measuring signal on substrate concentration results:

$$\Delta E \propto \text{const} \cdot \frac{1}{\beta} \cdot S$$

This equation shows that an enhancement of buffer capacity in the measuring cell gives rise to a progressive decrease of the slope of the calibration curve. At the same time the linear measuring range is shifted to higher urea concentrations. The initial pH of the buffer determines the sensitivity of the sensor, too. The pH optimum of kinetically controlled urea sensors is usually around pH 7.3 whereas a pH increase leads to a lower slope of the calibration graph. Moreover, it has to be taken into account that NH_3 and CO_2 formed from urea constitute a buffer system with a pK_a of 8.83. At high urea concentration this 'autobuffer' becomes effective; the calibration curve is therefore shifted to high substrate concentrations when a low initial pH is chosen.

2. Amperometric Electrodes

The advantages of amperometric electrodes, such as the greater sensitivity and precision and the lower measuring time, have prompted several research groups to study the adoption of this measuring principle to the assay of urea. Altogether four different approaches have been investigated:

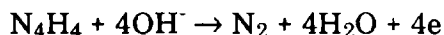
- (i) Urease and nitrifying bacteria have been sequentially coupled in a hybrid sensor, so that the NH_4^+ liberated by urease can be oxidized by the bacteria with consumption of oxygen (Okada et al. 1982, see Section 3.3.2.).
- (ii) The change of membrane permeability induced by pH shifts resulting from urea hydrolysis can be used to measure urea via amperometric indication of a permeant (Ishikara et al., 1985).
- (iii) A voltammetric ion sensitive electrode for ammonium ions based on indication of the ion transport through the interface between two immiscible liquids, e.g. ionophore-containing nitrobenzene and an aqueous solution, has been combined with immobilized

urease (Osakai et al., 1988).

- (iv) The linear dependence of the oxidation current of hydrazine on OH^- concentration (Fig. 69) has been utilized to follow the urease-catalyzed hydrolysis of urea (Kirstein et al., 1985a).

The sensor mentioned under (iii) uses an ammonium ion selective electrode carrying a PVC nitrobenzene-dibenzo-18-crown-6 gel. The enzyme was immobilized by crosslinking with glutaraldehyde on a Teflon membrane. After addition of urea to the measuring solution the ammonia produced in the enzymatic reaction reaches the internal solution layer (pH 9.7) of the sensor, where it is partially converted to ammonium ion which is transferred into the nitrobenzene phase. As a result, at 370 mV a current flow was observed which was related to urea concentration. The peak current was proportional to the concentration of urea between 1 $\mu\text{mol/l}$ and 2 mmol/l. The response time of the sensor was about 1 min.

The variant described under (iv) has been the most thoroughly studied. Between pH 5 and 9 the anodic oxidation current of hydrazine at +100 mV vs SCE (in the Tafel region) depends linearly on OH^- concentration:



In Fig. 70 this linear relation is compared with the logarithmic relationship obtained with potentiometric indication. The sensitivity depends on the initial pH. This is a considerable advantage over potentiometric methods in which only $dE/d\log a_{\text{OH}^-}$ is constant. The amperometric OH^- sensor has been coupled with a urease membrane (Kirstein et al., 1985a). The enzyme was entrapped in polyurethane or PVA. At low enzyme loading (4 U/cm^2) the pH-activity profile paralleled the pH profile of the enzyme, i.e., in the neutral region the sensitivity was at a maximum. The apparent K_M value of the urease membrane and that of the free enzyme were almost identical. Only above 17 U/cm^2 did the enzyme loading test display diffusion control of the amperometric urea sensor (Fig. 71). An apparent K_M of 32.6 mmol/l of this diffusion controlled urease membrane, together with an enhanced lifetime as compared with the kinetically limited sensor, indicate limitation by internal diffusion (Kirstein et al., 1985b). The response time of the amperometric urea sensor was 7–15 s, the sample frequency being 40/h, and the linear measuring range 0.8–50 mmol/l. The excellent precision was demonstrated by a CV below 1%. Since this measuring principle, like poten-

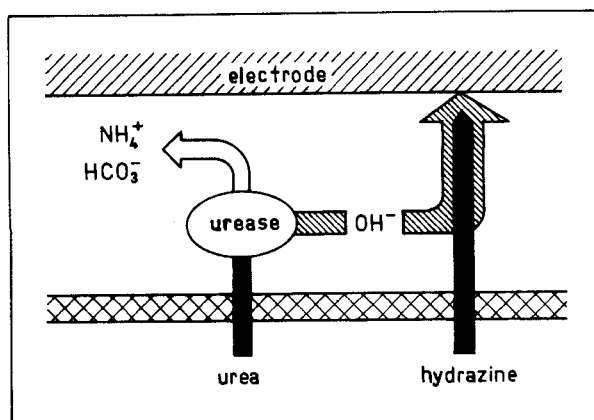


Fig. 69. Schematic of the processes in an amperometric urea electrode based on the pH dependence of the anodic oxidation of hydrazine.

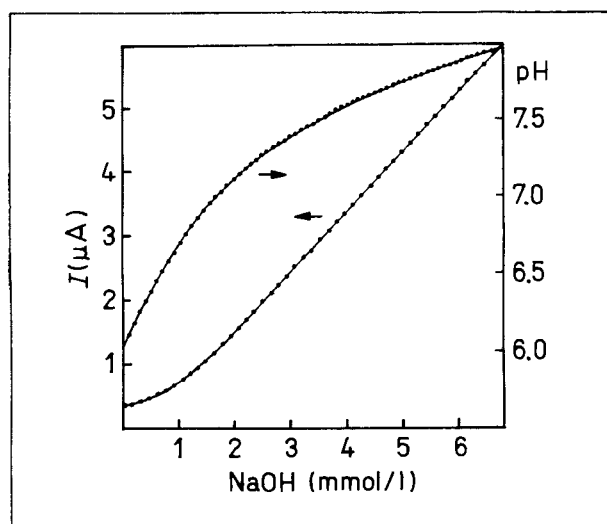


Fig. 70. Comparison of the signals of the amperometric urea electrode depicted in Fig. 69 (left axis) with those of a conventional pH glass electrode (right axis) on addition of sodium hydroxide to the measuring solution. (Redrawn from Kirstein et al., 1985b).

tiometric pH electrodes, is subject to influences by different pH values and buffer capacities of the samples, only a difference measurement with an enzyme-free electrode gives the true urea concentrations in serum.

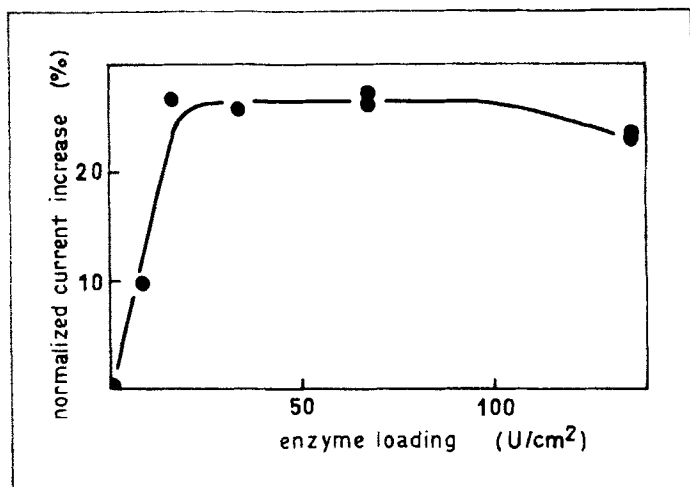


Fig. 71. Dependence of the stationary measuring signal of the electrode depicted in Fig. 69 on urease loading. Urea concentration: 0.25 mmol/l.

3. Conductometric Urea Sensors

The change in conductivity as a result of urea hydrolysis catalyzed by dissolved urease is evaluated in an analyzer available from Beckman (USA). The liberated NH_3 gas diffuses through a hydrophobic membrane to increase the specificity of the indication. The conductometric electrodes are surrounded by citrate buffer because this buffer type provides a strong increase in conductivity.

Lowe (1986) prepared conductometric electrodes by the screen-printing technique. The structure of the electrodes was deposited on a ceramic support by using gold or platinum ink, the printed support being subsequently fired to form metal electrodes. One pair of electrodes was covered by a polypyrrole solution containing urease and the pyrrole was polymerized anodically to give an insoluble film. Extremely small electrode arrangements can be produced using this technique. For compensation of nonspecific conductivity effects a pair of auxiliary electrodes was covered with pure polypyrrole. Calibration was carried out using appropriately buffered urea solutions to simulate the effect of buffer capacity of the medium.

The same research group described a small-scale electronic urea biosensor based on a urease-covered conductivity sensor (Watson et al., 1987/88). The conductometric sensor was prepared on a silicon wafer by applying the following sequence of steps: thermal oxidation, deposition

of titanium and platinum layers, photolithographic structuring, bonding, and encapsulation. Urease was attached to one pair of measuring electrodes in a mixture with BSA and glutaraldehyde. For measurement, an alternating current of 1 kHz and an amplitude of 10 mV was applied and the conductivity difference was evaluated against an electrode pair with no urease. The measuring signal obtained increased nonlinearly between 0.5 and 10 mmol/l urea. For serum measurement the samples had to be diluted 1:25 with imidazole buffer.

4. Optoelectronic Sensors

In analogy to optoelectronic glucose sensors (see Section 3.1.1.2), Lowe et al. (1983) developed a urea sensor based on the pH indicator bromothymol blue coimmobilized with urease on a transparent cellulose membrane. Within the first few minutes after sample addition the measuring signal increased linearly with time. The difference between the signals obtained one and two minutes after sample addition was evaluated by using a sample-and-hold differential amplifier. The linear range extended up to 10 mmol/l urea. The authors also developed a fiber optic urea sensor by fixing the above-mentioned membrane on the surface of a light-conducting fiber bundle. This principle was further developed by Arnold (1987) and Opitz and Lübbers (1987), who introduced pH dependent fluorescence indicators.

3.1.21.3 Biochemically Modified Devices

The Ir/Pd-MOS condensator described in Section 3.1.21.1 (Winquist et al., 1985) has also been combined with urease to form a urea probe. Urease was entrapped between a dialysis membrane and an NH_3 -permeable foil, the latter being separated from the MOS structure by an air gap of 0.1 mm width (Fig. 72). The measuring range was 0.01–5 mmol/l, the response time being 3 min. The sensor had an operational lifetime of only 4 days.

Anzai et al. (1985) constructed a microsensor for urea assay by immobilizing urease to the gate of a pH sensitive FET. The enzyme was mixed with glutaraldehyde and crosslinked at an Si_3N_4 gate. The measurements were performed in constant drain mode by using an external calomel electrode. In unstirred solution of 0.1 mmol/l phosphate buffer, pH 6.0, the sensor exhibited a slope of 50 mV/decade between 0.5 and 20 mmol/l urea. High urea concentrations drastically reduced the sensitivity by raising the pH in the enzyme layer.

Immobilization of urease to a silanized Si_3N_4 gate by glutaraldehyde

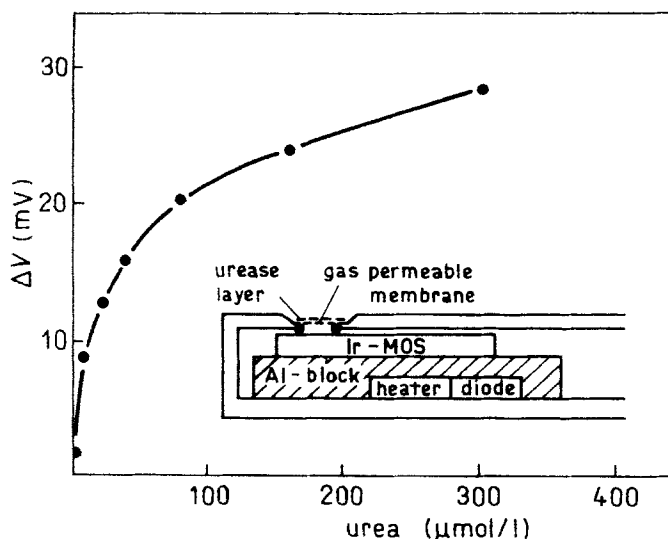


Fig. 72. Concentration dependence of the stationary measuring signal of a urease-covered Ir-MOS condensator. (Redrawn from Winqvist et al., 1986).

vapor has provided an enzyme-FET with a functional stability of 20 days and a response time of only 30 s (Karube et al., 1986).

Enzyme-FET systems for glucose and urea, in which the pH within the enzyme layer is kept constant by electrolytic production of H^+ or OH^- ions, have been described by Van der Schoot and Bergveld (1987/88). By the application of direct feedback, this pH-static sensor becomes independent of the buffer capacity and pH of the sample solution; the response is linear, and the dynamic range can be expanded.

Nakamoto et al. (1987) developed a technique for covering the gate with an enzyme layer (Fig. 73). A wafer containing two pairs of pH-FETs was coated with a positive photoresist and the gate region exposed to light. After developing, the gate was silanized and covered with urease solution containing glutaraldehyde by spin coating. After 30 min the photoresist was removed from the wafer by sonication in acetone. The authors did not report any measures to protect the enzyme during this procedure. With a response time of only 5 s the urea sensor was extremely fast.

Urease has also been adsorbed to the surface of a Langmuir-Blodgett film and the film transferred to the gate of an ISFET (Karube 1986; Moriizumi and Onoue, 1986).

Another urea microbiosensor, based on an iridium oxide electrode,

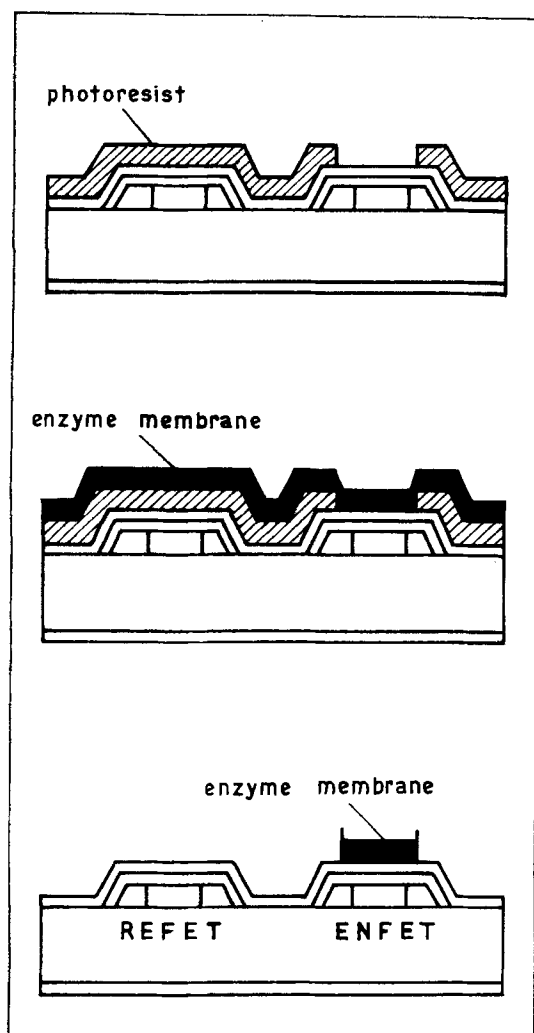


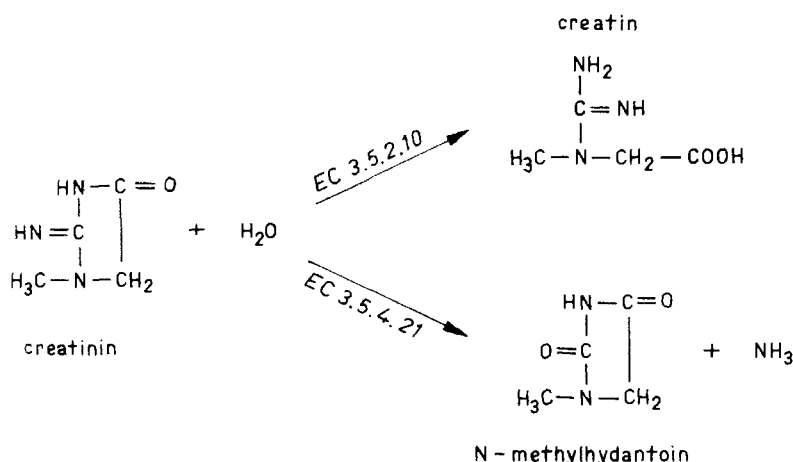
Fig. 73. Schematic representation of the lift-off method for enzyme coating of FETs. (Redrawn from Nakamoto et al., 1987).

has been developed by Suva et al. (1986). The electrode surface was covered by a polymer layer of 40 nm thickness containing carbonyl groups to which urease had previously been bound covalently. An apparent enzyme activity of 40 mU/cm^2 was obtained. The sensor reached 90% of the steady measuring signal within 4 s after sample injection, the response time being strongly dependent on stirring speed. In 10 mmol/l phosphate buffer the signal for 5 mmol/l urea was as low

as 3 mV. Within 24 h the sensitivity decreased by 20–40%.

3.1.22 Creatinine Sensors

For the diagnosis of kidney function, creatinine determinations in serum are increasing in popularity over urea measurements, since the creatinine level is not affected by a high protein diet or by metabolic rate, as is the case for urea. Enzymatic methods use either creatinine amidohydrolase (EC 3.5.2.10) or creatinine iminohydrolase (EC 3.5.4.21):



The reaction products, e.g. creatine, can be assayed by coupling the creatine kinase, pyruvate kinase, and LDH systems. The reduction of the absorbance of NADH is measured and equated to the concentration of creatinine. The enzymes must be rather pure, which makes the procedure very expensive. Since the physiological level of creatinine is as low as 100 μmol , the values obtained have to be corrected for pyruvate and creatine.

The combination of the creatinine-converting enzymes with sensors indicating primary reaction products, such as ion sensitive electrodes, NH_3 gas sensors, or thermistors, is an effective alternative to enzyme sequence sensors (see Section 3.2.1). Enzyme reactors as well as 'true' biosensors for creatinine have been described.

Danielsson (1982) proposed a reactor with immobilized creatinine iminohydrolase in a thermistor unit. Although the measuring range was 0.01–10 mmol/l creatinine, the sensitivity was still too low for the assay

of diluted serum samples. The enzyme has also been coupled in a flow system with an ammonia gas-sensing MOS transistor, the gate of which was modified by a thin iridium layer (Winqvist et al., 1986). This Pd/Ir-MOS sensor must be operated in the gas phase, i.e., it has to be separated from the aqueous solution by means of a gas-permeable membrane. In order to eliminate endogenous NH_3 , which would also be indicated by the sensor, the authors introduced a reactor containing immobilized glutamate dehydrogenase upstream of the creatinine iminohydrolase reactor. The detection limit of this system was $0.2 \mu\text{mol/l}$, so that strong dilution of plasma and urine samples was possible. Fifteen samples per hour could be assayed with a CV of 3.4%.

The very same enzymes have been combined by Mascini et al. (1985a) in an FIA system. Creatinine iminohydrolase was immobilized on the inner wall of nylon tubing (diameter 1 mm, length 1 m) and the ammonia liberated in the enzymatic reaction was measured with an NH_3 electrode. Owing to the low sensitivity of the indicator electrode, the linear range was only 0.01–0.2 mmol/l.

Meyerhoff and Rechnitz (1976) developed a potentiometric creatinine sensor by inclusion of creatinine iminohydrolase between the gas-permeable membrane of an ammonia electrode and a dialysis membrane. Since the specific activity of the enzyme used was very low, 0.1 U/mg, only 43 mU could be entrapped at the electrode. Therefore the sensor was kinetically controlled and reacted to addition of the enzyme activator tripolyphosphate by an increase in sensitivity from 44 mV to 49 mV per concentration decade and a corresponding decrease of the detection limit. These effects agree with theoretical considerations of reaction–transport coupling. The samples were treated with a cation exchanger to remove endogenous serum ammonia.

Guilbault and Coulet (1983) achieved the sensitivity required for the assay of physiological creatinine concentrations by using creatinine iminohydrolase from *Clostridium paraputrific*, which has a higher specific activity, 1.9 U/mg. The enzyme was attached to collagen or small intestine membranes by the acyl-azide method. The resulting membranes were fixed to an NH_3 probe. The sensor was stable for 20 days or 100 determinations. The electrode potential was proportional to the logarithmic values of creatinine concentration over the range of 1 to 100 mg/l. When the NH_3 level of the sample was above normal (24–48 $\mu\text{mol/l}$), a difference measurement with an electrode without enzyme was necessary.

3.1.23 Penicillin Sensors

Penicillins are the world's most important antibiotics, the production per year being worth \$25 million. The quantitation of penicillin is required for control of the fermentative production of penicillin G and penicillin V and their splitting to 6-aminopenicillic acid (6-APA). 6-APA is the basic material for semisynthetic penicillins.

β -Lactamase (EC 3.5.2.6), also called penicillinase, splits the lactam ring of the penicillins with the formation of penicilloic acid. The acid, which competitively inhibits the enzyme, has a pK around 3.0. Penicillin amidase (EC 3.5.1.11) catalyzes the hydrolysis of the amide bond in position 6 with the liberation of 6-APA and an organic acid (Fig. 74).

These reactions are coupled with a signal transducer using mainly the pH decrease and the reaction enthalpy. Whereas β -lactamase has been combined with various transducer types, penicillin amidase has been applied only in one enzyme electrode for penicillin (Table 9).

3.1.23.1 Enzyme Reactors

Decristoforo and Danielsson (1984) designed an enzyme thermistor for penicillin determination based on β -lactamase immobilized on porous

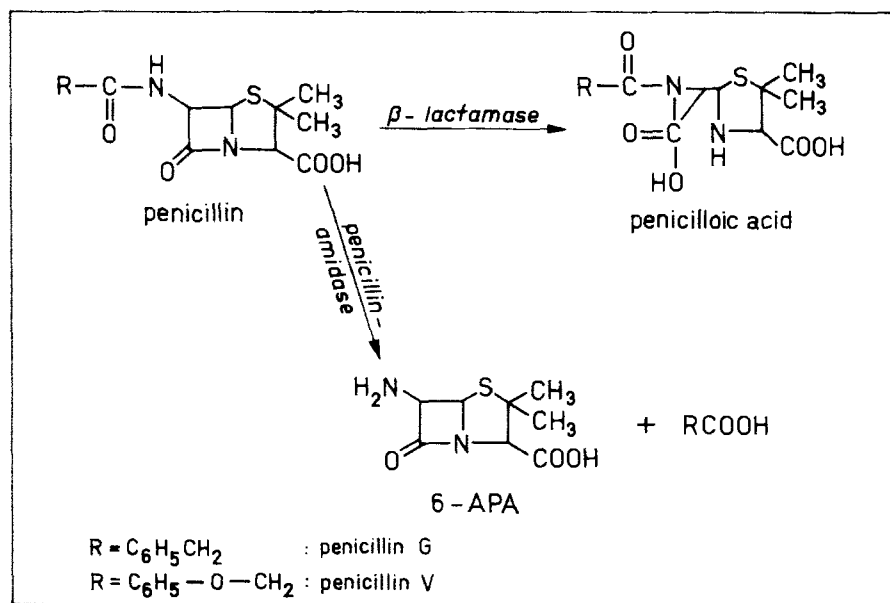


Fig. 74. Analytically utilized reactions of penicillin cleavage.

TABLE 9
Biosensors for Penicillin

Base sensor	β -Lactamase	Amidase	Linear range (mmol/l)	Lifetime (d)	Sample frequency (h ⁻¹)	CV (%)
Thermistor	reactor		0.1–100	20	40	2
Optoelectronic	immobilized		0.5–5	20	12	2
pH electrode	immobilized		0.08–0.5	30	10	5
pH electrode	immobilized		1–20	50	20	
pH electrode		immobilized	1–20	10	20	
pH electrode	free		1–30	7		
pH electrode	free		1–10	10	10	5
pH electrode	reactor		0.05–0.5	250	150	
Sb microelectrode	immobilized		0.3–7	10	60	
pH-FET	immobilized		5–50		20	

glass. The device was able to measure penicillin in fermentation broth in the range 0.1–100 mmol/l in good agreement with the chemical method. Continuous measurement of penicillin as well as cephalosporin has been demonstrated in aqueous standard solutions and fermentation samples.

Excellent results have been obtained by the combination of a bead string reactor containing β -lactamase with a flat glass electrode in an FIA system (Guanasekaran and Mottola, 1985). The enzyme was bound by glutaraldehyde crosslinking to the silanized walls of the reactor and to glass particles contained in the reactor. This reactor had outstanding flow properties and permitted the processing of 150 samples per hour.

Olsson (1988) obtained a pH signal which was linearly dependent on penicillin concentration by using a penicillinase reactor in an FIA manifold. The linearity was due to complete substrate hydrolysis in the reactor and an almost constant buffer capacity of the carrier stream. To exclude disturbances from varying sample pH the penicillin concentration was calculated as the difference between the response with and without hydrolysis.

3.1.23.2 Membrane Sensors

β -Lactamase has been coimmobilized with the pH indicator bromocresol green on a transparent cellulose membrane in a flow-through device (Lowe and Goldfinch, 1983). This reagentless system responded linearly to penicillin in the range 0.5–10 mmol/l. With fermentation samples, however, disturbances caused by varying pH values and buffer capacities of the samples have to be expected.

Fuh et al. (1988) devised an enzyme optrode for penicillin. β -Lactamase was immobilized on a fluorescein isothiocyanate-labeled porous glass particle which was glued to the tip of a fiber optic bundle. Excitation was carried out by an argon laser. pH changes resulting from the enzyme reaction led to changes of the fluorescence intensity. The response time of the sensor was 20–45 s, the detection limit being 0.1 mmol/l penicillin.

Potentiometric penicillin sensors are mainly based on glass electrodes working with free enzyme or with enzyme included in an electrode cover. In the first penicillin electrode, β -lactamase was directly photopolymerized in acrylamide on the electrode (Papariello et al., 1973). Enfors and Nilsson (1979) designed a sterilizable penicillin sensor where a solution containing β -lactamase was pumped into a reaction chamber in front of a flat glass electrode, after sterilization of the electrode (Fig. 75).

A thin-layer antimony oxide electrode generated by vacuum evaporation on ceramic has been employed as a pH transducer in a penicillin sensor designed by Flanagan and Carrol (1986). Whereas evaporation of antimony onto a glass support gave rather insensitive pH sensors, Sb-layers on aluminum oxide exhibited a pH function of 59 mV/decade. These layers adhere very well to the support. Though providing a well-adhering monolayer, coupling of the enzyme by carbodiimide decreased the stability to 2–3 days. This kind of layer probably becomes detached together with the hydrated Sb_2O_3 layer. Best results have been

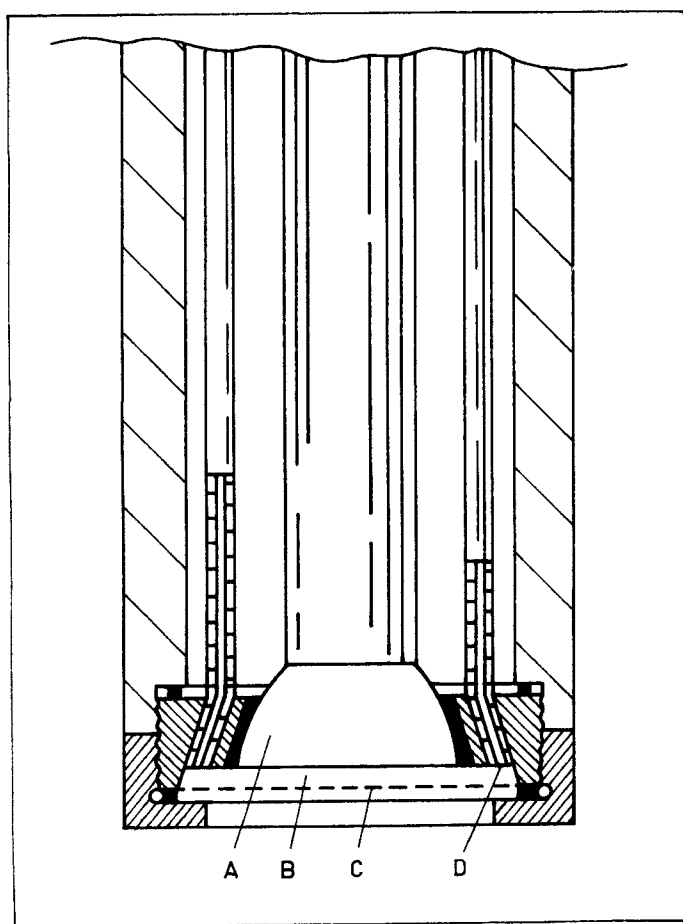


Fig. 75. Construction of a sterilizable enzyme electrode for the determination of penicillin. A: flat glass electrode, B: reaction chamber containing enzyme, C: dialysis membrane, D: holder. (Redrawn from Enfors, 1987).

obtained by crosslinking the enzyme with glutaraldehyde together with BSA. This layer adheres tightly and provides a linear dependence for penicillin G in the range 0.3–7 mmol/l on a semilogarithmic plot. In stirred solution the steady state potential was reached within 30–60 s. This penicillin sensor was stable for 10 days, after which the sensitivity decreased strongly, probably due to inactivation of the immobilized β -lactamase. Nevertheless, the stability is high enough to control a complete penicillin fermentation cycle.

Vacuum evaporation is cheap and enables the creation of multiple sensors on small surfaces. It provides therefore a good alternative to ISFET technology in biosensors. Another advantage is the possibility of using established methods for enzyme immobilization.

3.1.23.3 Enzyme Field Effect Transistors

The first enzyme-FET, proposed by Caras and Janata in 1980, was based on a pH-FET bearing immobilized β -lactamase. The gate was covered with a mixture of enzyme and BSA which was subsequently crosslinked by glutaraldehyde. In addition to the enzyme-FET a common pH-FET was combined with a reference electrode to compensate for inconstant sample pH. This configuration initiated the development of microbiosensors for substrate as well as antibody determination.

In more recent studies, Janata et al. (1985) derivatized β -lactamase with N-succinyl methacrylate prior to immobilization. In order to achieve good adhesion the Si_3N_4 gate was pretreated with BSA and glutaraldehyde. The modified enzyme was transferred in polyacrylamide onto the gate region and polymerized. The sensor had a linear range of 5–50 mmol/l with a response time of 60 s. An enzyme-free pH-FET on the same chip served to measure the pH change resulting from sample injection, which was subsequently subtracted from the enzyme-FET signal.

Anzai et al. (1987) and Anzai and Hashimoto (1988) succeeded in applying the Langmuir-Blodgett (LB) technique to cover the gate of an ISFET with penicillinase. In the first step, between 1 and 100 monolayers of stearic acid were deposited by immersing the gate region into the subphase. After drying the LB multilayer the ISFET was immersed in a 0.5% penicillinase solution. The prepared sensor could be used to determine penicillin G for more than 15 days without deterioration of the response.

3.1.24 Determination of Glycerol and Triglycerides

Triglycerides, together with cholesterol and phosphatides, constitute the most important lipid fraction of blood. They are localized in the chylomicrons and the very-low-density lipoproteins. The established methods of triglyceride assay require long preincubation and various enzymes such as lipase, glycerokinase, pyruvate kinase, and lactate dehydrogenase. The design of methods for direct indication of the primary products of the enzyme-catalyzed triglyceride conversion is therefore an important task in clinical chemistry.

Satoh et al. (1981) have shown that triglyceride assay is possible with a combination of a column of immobilized glycerol ester hydrolase (lipase, EC 3.1.1.3) and a thermistor. The enzyme was immobilized to silanized porous glass according to the method of Weetall (1976). The highest immobilization yield was achieved by using carriers with the lowest possible pore diameter (73 nm), i.e., they had the largest internal surface. On the other hand, the measured activity was substantially higher when carrier material with large pores (220 nm) was employed. This was evidently due to steric hindrance caused by the large substrate molecules. By investigating the influence of different buffer solutions the authors found that the ester hydrolysis itself produces only little heat whereas buffers with high protonation enthalpy, e.g. Tris, support a high signal. Under these conditions satisfactory sensitivity was obtained for triglyceride determination in serum samples of 0.5 ml. The calibration curve was linear between 0.1 and 5 mmol/l glyceryl trioleate and the CV was 4%. The enzyme thermistor was calibrated with triglyceride standard solutions containing 4.5% human serum albumin and 0.5% Triton X 100 before measuring actual serum samples. The results agreed well with those obtained by the enzymatic-spectrophotometric method.

The free fatty acids liberated in glycerol ester hydrolysis can also be measured by using a glass electrode. Satoh et al. (1979) coupled a reactor containing lipase immobilized on polystyrene with a flow-through glass electrode. For assay of serum samples the neutral fat was extracted with isopropanol and the organic phase was injected directly into the carrier stream of 0.5 mmol/l Tris-HCl, pH 7. The measuring time for one sample was 3 min. Good agreement was found with the spectrophotometric procedure.

Recently, the glass electrode has been replaced by two pH-FETs working in a differential circuit, lipase being fixed to one of the gates

(Nakako et al., 1986). The triglycerides were solubilized by 10% Triton X 100. The measuring range of the sensor was one concentration decade, the response time being 2 min.

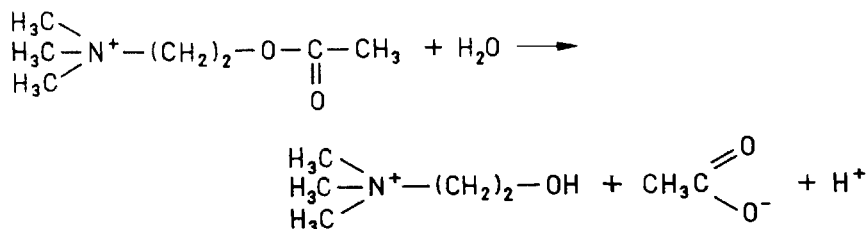
Since the use of detergents in conjunction with immobilized enzymes generally causes problems, none of the methods described has been routinely applied in clinical chemistry.

As an alternative to the indication of pH change, glycerol formed during the hydrolysis of triglycerides can be measured. For this purpose glycerol oxidase, a novel enzyme isolated from *Aspergillus*, (Uwajima et al., 1984) might be useful; it has not yet been employed in enzyme electrodes. Fonong (1987) proposed the use of glycerol dehydrogenase (EC 1.1.1.6) and measurement of the NADH formed by anodic oxidation at a potential of +0.7 V. The enzyme was covalently bound to collagen, and the sensor exhibited a linear calibration curve between 0.2 and 12 $\mu\text{mol/l}$ glycerol.

3.1.25 Determination of Acetylcholine

Acetylcholine is the most important neurotransmitter. Its release into the synaptic cleft triggers the opening of ion channels resulting in the generation of an action potential. The rapid degradation of acetylcholine (ACh) as catalyzed by acetylcholinesterase (AChE, EC 3.1.1.7) provides regeneration of the initial state. These functions make the determination of ACh as well as AChE and its inhibitors, some of which find application as insecticides and chemical warfare agents, highly important in clinical chemistry and environmental control.

Acetylcholinesterase hydrolyzes ACh to choline and acetate. One mole of H^+ is formed per mole of ACh by dissociation of acetate:



The increase of the concentration of H^+ can be indicated by means of glass electrodes. Durand et al. (1978) entrapped AChE around the active tip of a glass electrode in a gelatin layer of 50 μm thickness and, after drying, crosslinked the layer with glutaraldehyde. In 0.01 mol/l phos-

phate buffer, pH 8, a linear dependence of the electrode potential on ACh concentration between 0.1 and 2 mmol/l was found. The sensor was stable for several weeks. In a similar manner, Suaud-Chagny and Pujol (1985) designed a miniature enzyme electrode for ACh.

3.1.26 Determination of Sucrose

Invertase (β -D-fructofuranosidase, EC 3.2.1.26) cleaves sucrose to give fructose and glucose. As in the assay of other disaccharides, the glucose can be measured in a coupled enzyme reaction. No coupled reaction is required in an enzyme thermistor based on immobilized invertase as described by Mandenius et al. (1981). This device was stable for 6 months and responded linearly to sucrose concentrations between 0.05 and 100 mmol/l. No signal was obtained for glucose and fructose. The equipment has been combined with a continuous sampling system consisting of a CO₂ degassing unit and a diluter for on-line sucrose measurement in alcohol production by immobilized yeast.

3.2 BIOSENSORS USING COUPLED ENZYME REACTIONS

Since not all enzyme-catalyzed reactions involve transducer-active compounds such as H⁺, oxygen, or hydrogen peroxide, only a limited number of substances can be determined by using monoenzyme sensors. Owing to their low sensitivity to the analyte as compared with that towards nonspecific effects, the application of transducers which indicate general reaction effects, e.g. thermistors or piezoelectric detectors, is not always appropriate as well. In such cases coupled enzyme reactions for analyte conversion provide a favorable alternative. The primary product of the conversion of the analyte is further converted enzymatically with the formation of a measurable secondary product or an otherwise greater reaction effect. Whereas the biocatalyst responsible for the primary reaction should be rather specific, the coupled reaction usually requires only a group-specific enzyme. Whole families of sensors have been developed on the basis of such *sequentially coupled* enzymes, combining glucose-, lactate-, or alcohol-producing primary enzymes with the respective oxidases (Fig. 76).

In addition to such an extension of the spectrum of measurable compounds, enzyme sequences are also useful to enhance the selectivity of indicator reactions, e.g., in GOD-HRP electrodes, and to enhance the sensitivity, e.g., in thermistors using a GOD-catalase sequence. These

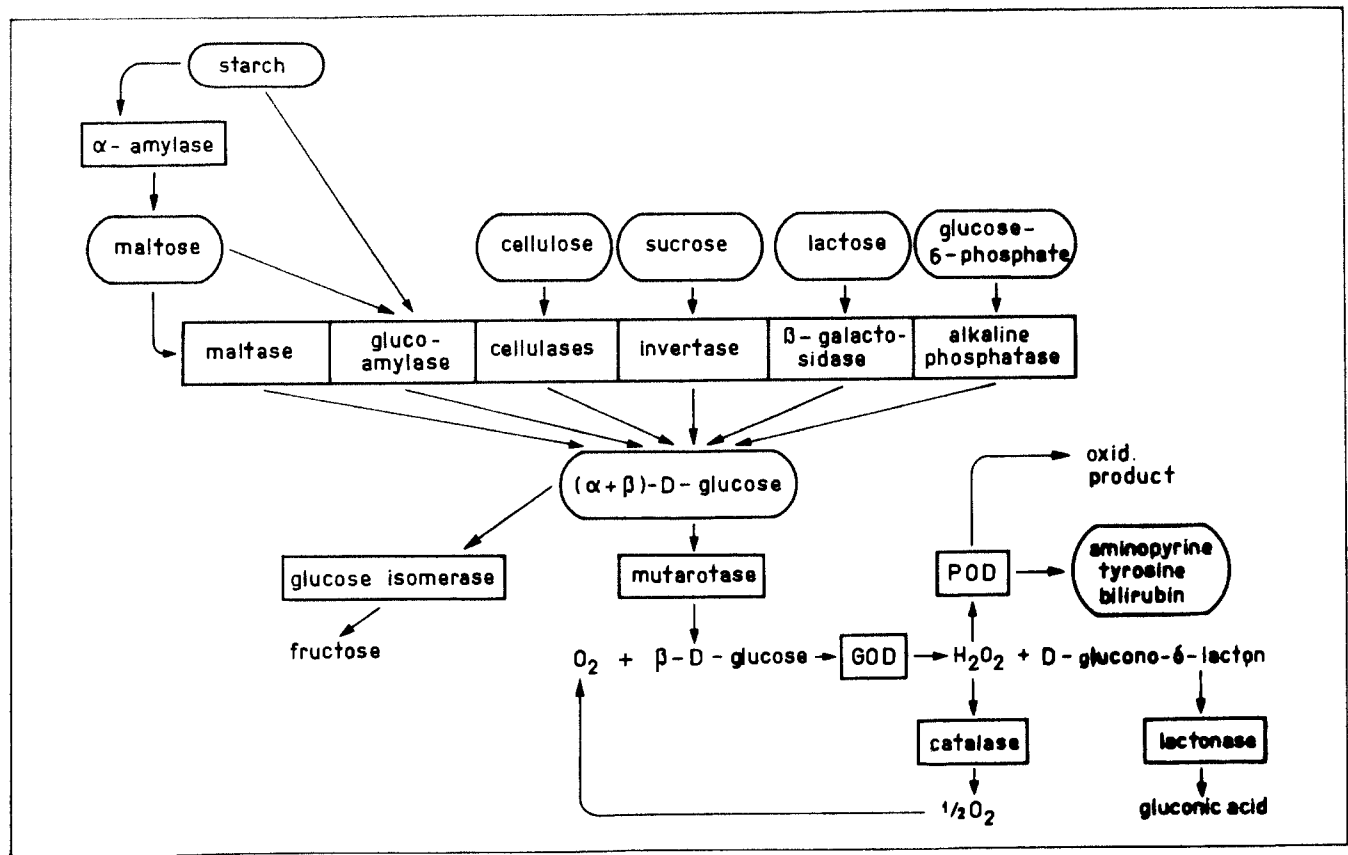


Fig. 76. Enzymes sequentially coupled with GOD for the extension of GOD sensors to other analytes.

types of sequential coupling resemble those occurring in metabolism, where energy-rich substrates are degraded in a stepwise manner, as in glycolysis, photosynthesis, or the citrate cycle, and enzyme cascades are responsible for signal amplification in many receptor systems. In the latter, the chemical modification of enzymes leads directly to a snowballing increase of the reaction rate. So far, this powerful principle has not been used in biosensors; however, the so-called 'apoenzyme electrodes', in which the recombination of an apoenzyme with its prosthetic group results in a dramatic enhancement of the reaction rate, may be considered to be an analogue.

Another type of sequential coupling is provided by *cycling reactions*. The product of the primary enzyme reaction is regenerated to the substrate of this reaction, i.e., the analyte, in a second, enzyme-catalyzed reaction. These cycles are based on the dependence of the two enzymes on different cofactors; thus, the required free enthalpy exists for both reactions. The analyte molecule may be regarded as a catalyst of the reaction between the two cofactors. This results in a rate of cofactor conversion and enthalpy production that is enormously higher than that in a single enzyme reaction. These cycling reactions therefore lead to a substantial increase of sensitivity.

The principle of signal amplification by using the free enthalpy of energy-rich compounds forms the basis of signal processing in nervous systems. Although the signal transmission makes use of mechanisms that are different from those mentioned above, recognition and signal amplification are also based on enzyme reactions. On the other hand, substrate cycles, such as the glucose-6-phosphate cycle in glycolysis, are important for rapid energy supply.

Parallel coupling constitutes another type of coupled enzyme reactions. This includes the competition of two enzymes for a common substrate as well as the conversion of alternative substrates and the competitive binding of a substrate and an inhibitor to an enzyme. Thus, analytes become measurable even though they cannot be converted to readily detectable products. Coupled enzyme reactions can also be used to eliminate disturbances of the enzyme or transducer reaction caused by constituents of the sample. Compounds interfering with the signal transduction can be transformed into inert products by reacting them with an (eliminator) enzyme which can be coimmobilized with the analyte-converting (indicator) enzyme in the vicinity of the transducer. On the other hand, constituents of the sample which are at the same time intermediate products of coupled enzyme reactions and will thus

be indicated together with the analyte can be eliminated before they reach the indicator enzyme layer. For this purpose, several enzyme layers have to be employed to avoid conversion of the intermediate product formed in the indicator enzyme layer to an inactive substance. Therefore the 'anti-interference layer' has to be arranged on the solution side of the sensor.

3.2.1 Enzyme Sequence Sensors

As early as 1965, Clark proposed a bi-enzyme electrode for the determination of sucrose composed of coimmobilized invertase and GOD. Later on the concept of coupling one or more hydrolases or lyases with an oxidase was expanded to sensors for other disaccharides and for polysaccharides, creatinine, adenosine monophosphate, and esters of glycerol, choline, and cholesterol. Transferases have been sequentially coupled with oxidases or dehydrogenases to form the hexokinase + glucose-6-phosphate dehydrogenase, alanine aminotransferase + glutamate dehydrogenase, and pyruvate kinase + LDH + LMO systems. In each case the oxidoreductase-catalyzed reaction liberates the product to be indicated at the transducer. The coupling of oxidoreductases with each other has been achieved by using GOD and catalase or HRP, respectively. The aim of this combination is an improvement of the transducer reaction.

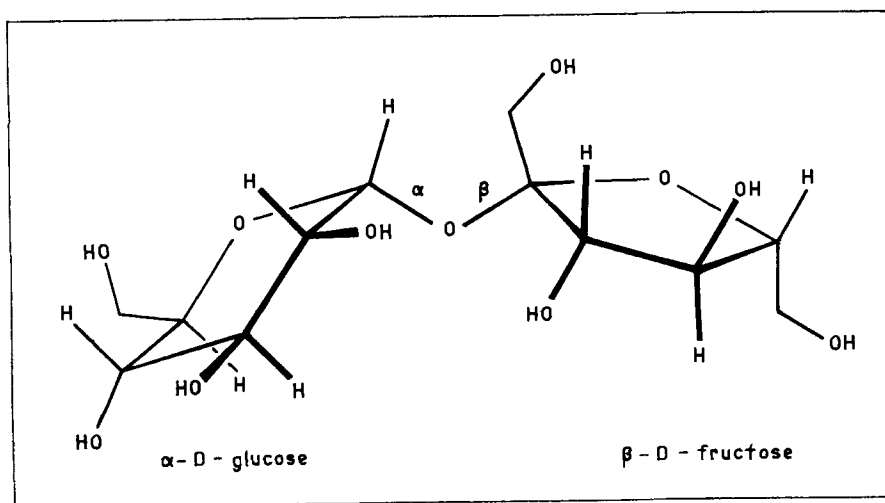
In potentiometric enzyme electrodes lyases producing carbon dioxide or ammonia are used as terminal enzymes of sequences. In fact, the term 'enzyme sequence electrode' was introduced on the occasion of the design of a potentiometric D-gluconate sensor containing gluconate kinase (EC 2.7.1.12) and 6-phosphogluconate dehydrogenase (EC 1.1.1.44) (Jensen and Rechnitz, 1979). The authors found that for such a sensor to function the optimal pH values of the enzymes and the transducer should be close to each other. Furthermore, cofactors, if necessary, must not react with one another nor with constituents of the sample. It was concluded that the rate of substance conversion in multiple steps cannot exceed that of the terminal enzyme reaction. A linear concentration dependence is obtained when an excess of all enzymes of the sequence is provided, i.e. complete conversion occurs of all substrates within the enzyme membrane. Different permeabilities of the different substrates results in different sensitivities. This is particularly important with combinations of disaccharidases and oxidases, where the substrate is cleaved to two monosaccharides of approximately the same molecular size. The above

authors suggested immobilizing sequentially acting enzymes by mechanical entrapment between two membranes in order to assure a high mobility of the intermediates.

3.2.1.1 Enzyme Sequence Sensors for Disaccharides

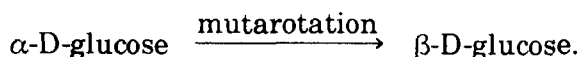
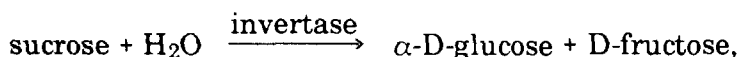
In biosensors for the determination of sucrose, lactose, and maltose, invertase (EC 3.2.1.26), β -galactosidase (EC 3.2.1.23), maltase (EC 3.2.1.20), or myrosinase (EC 3.2.3.1) are coupled sequentially with GOD. Since the hydrolysis of disaccharides does not lead to an equilibrium mixture of α - and β -glucose, the inclusion of mutarotase (EC 5.1.3.3) has proved useful. Oligo- and polysaccharides can be assayed by coupling glucoamylase (EC 3.2.1.3) or cellulase (EC 3.2.1.4) with GOD.

The determination of sucrose in the microbiological and food industry is of a similar importance as the determination of glucose in the clinical laboratory. Sucrose consists of β -D-fructose and α -D-glucose linked to each other by the glycosidic OH-groups:



Various research groups have developed enzyme electrodes for the determination of sucrose. The operational parameters of these sensors are listed in Table 10.

In invertase-GOD electrodes glucose is formed in the following sequence of reactions:



With an excess of invertase and GOD in the enzyme membrane the total rate of sucrose determination is limited by the spontaneous mutarotation. Therefore the sensitivity towards sucrose is only about 10% of that for glucose (Scheller and Karsten, 1983). Kinetic (dI/dt) measurement even gives only 1% of the glucose signal at the same sucrose concentration. Application of coimmobilized mutarotase gives rise to an increase of the sensitivity by a factor of 6 for stationary measurement

TABLE 10

Enzyme Electrodes for Sucrose

Enzyme			Range (mmol/l)	CV (%)	Stability	Sensitivity	References
GOD	Mutarotase	Invertase				glucose/ sucrose	
x	x	x	0.1–2.5	7	10 days		Satoh et al. (1975)
x		x	1.4–14			10	Cordonnier et al. (1975)
x	x	x	0.1–2				Bertrand et al. (1981)
x	x	x		1.8			Mason et al. (1983a)
x	x	x			18 days	2	Kulys et al. (1979)
x	x	x	0.03–1.5	3–4	1 week	1.7	Macholán et al. (1983)
x		x	0.5–70	3	1 week	16	Scheller and Karsten (1983)
x	x	x	0.5–7	3	4 days	3	Scheller and Karsten (1983)

and by a factor of 60–100 for rate measurement. In this manner, sucrose can be assayed with 60% of the glucose sensitivity.

Although the substrate specificity of invertase is very high (apart from sucrose only raffinose is hydrolyzed), sucrose sensors suffer from a lack of selectivity because they also indicate the endogenous glucose contained in most samples. Employment of two measuring devices enables the parallel determination of glucose and sucrose by using a GOD electrode and a trienzyme electrode, both inserted in a double-measuring cell (see Fig. 27). In contrast, with only one analyzer, the enzyme membranes have to be exchanged during the measuring process. In this case it is advisable to hydrolyze the sucrose with invertase and subsequently measure the sum of glucose and sucrose by means of a GOD-mutarotase sensor (Fig. 77) (Scheller and Karsten, 1983). Five units of invertase and a preincubation time of 5 min have been shown to be sufficient for complete hydrolysis. The inversion can also be carried out directly in the measuring cell. After reaching the stationary current signal for endogenous glucose, dissolved or immobilized invertase is injected and the rate of sucrose cleavage indicated (Fig. 78) (Weise and Scheller, 1981; Scheller and Karsten, 1983). The rate of glucose liberation depends linearly on the sucrose concentration. This procedure is restricted to samples containing minor amounts of glucose. Interference

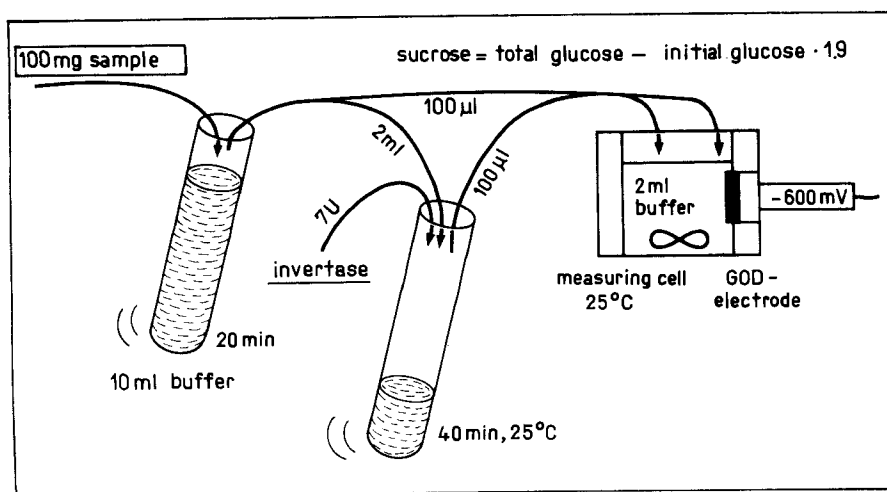


Fig. 77. Procedure of total glucose determination by external splitting of sucrose by invertase.

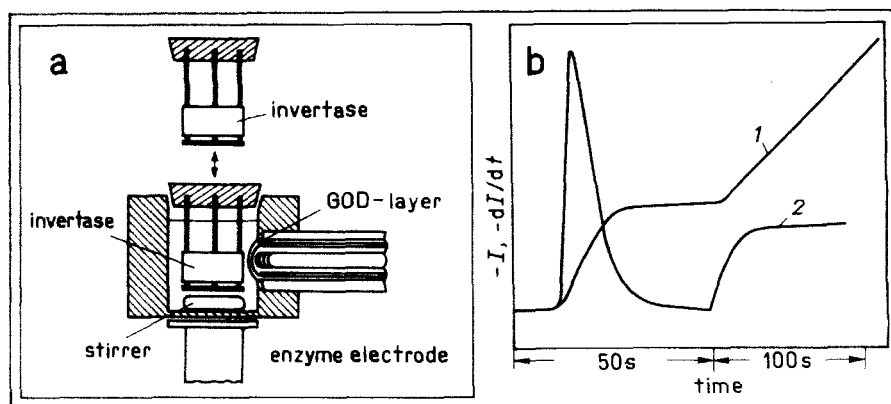


Fig. 78. Determination of sucrose with immobilized invertase and a GOD electrode. (a) Measuring cell. (b) Measuring curves of successive assay of glucose and sucrose. 1: current indication (I), 2: kinetic indication (dI/dt).

TABLE 11

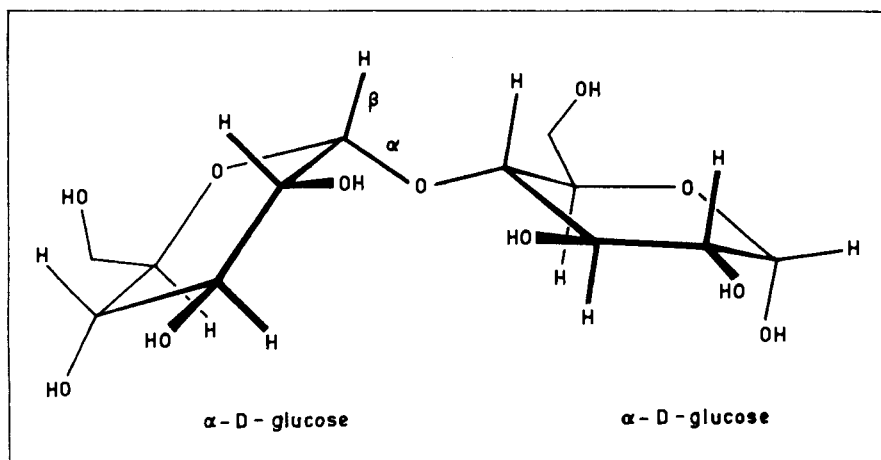
Comparison of Different Procedures for the Determination of Glucose and Sucrose

	External inversion	Internal inversion	Trienzyme electrode	Anti-interference electrode
CV (%)	1.9	5.0	3.8	3.2
Measuring range (mmol/l)	0.5–44.0	0.5–12.0	0.5–7	0.5–20.0
Sample frequency (h^{-1})	10–12	6	20	8

by higher glucose concentrations can be eliminated by affixing a GOD-catalase membrane in front of the invertase-GOD membrane. A more detailed description of this principle will be given in Section 3.2.3.

The results obtained by the different variants of sucrose assay in glucose-containing samples as performed with the Glukometer analyzer (ZWG, GDR) are summarized in Table 11.

Maltose consists of two glucose units linked via a (1-4)- α -D-glycosidic bond:



Under equilibrium conditions 39% is present as α -maltose, having the free glycosidic hydroxyl group in the α -configuration. Since degradation of starch by α -amylase primarily yields maltose, assay of maltose is of relevance in the control of brewery and distillery processes.

Cleavage of maltose by maltase leads to a fraction of β -D-glucose which is considerably lower than that present in equilibrium (63%). Consequently, the sensitivity of a maltase-GOD sequence electrode is lower for maltose than for glucose. Such a sensor is being used in a commercial analyzer for α -amylase assay in serum (Osawa et al., 1981), indicating the rate of maltose formation.

The α -glycosidic bond of maltose is also split by glucoamylase. In this reaction only the β -form of the free glycosidic OH-group is produced. Thus the hydrolysis of maltose gives 26% α -glucose and 74% β -glucose or, in other words, 1 mmol/l maltose gives 1.48 mmol/l β -D-glucose whilst a glucose solution of 1 mmol/l contains only 0.63 mmol/l β -D-glucose. A glucoamylase-GOD electrode may therefore be expected to be more than twice as sensitive for maltose as it is for glucose. In a study of such a sensor, Scheller et al. (1983b) found that the sensitivities were actually almost identical, and they obtained a linear range up to a maltose concentration of 2 mmol/l. Under internal diffusion limitation the excess of β -D-glucose is obviously overcompensated by the higher diffusion resistance of the membrane for maltose. The pH optimum of this sensor was determined to be 5.6 for glucose (as that of the corresponding glucose electrode) and 4.5 for maltose. A compromise pH of 5.0 is chosen for the measurements. A high glucoamylase concentration (50 U/cm²) is necessary in order to obtain diffusion control for maltose.

In addition to glucose and maltose the glucoamylase–GOD electrode measures oligosaccharides, dextrans, and soluble starch. Although the activity of glucoamylase increases with increasing chain length of the substrate, the sensitivity decreases for high-molecular weight substrates. This is also due to the dominating influence of the diffusion resistance. The different sensitivities towards different saccharides preclude application of the sensor to an assay of 'total glucose' in complex media such as fermentation broths. External hydrolysis by glucoamylase may circumvent this problem. The glucose formed can then be measured with a GOD–mutarotase electrode.

Lactose is a constituent of milk, human milk containing 0.3–0.6 mol/l, and bovine milk 0.25–0.28 mol/l. Lactose consists of 1,4-glycosidically linked β -D-galactose and D-glucose.

The possibility of assaying lactose by using a galactose oxidase sensor was pointed out in Section 3.1.2. A higher specificity can be achieved with the β -galactosidase–GOD sequence. A sensor based on this system has been described by Pfeiffer et al. (1987). With a linear range up to 4 mmol/l lactose the sensitivity of this bienzyme electrode was only 60% of that for glucose. With both enzymes entrapped in gelatin and cross-linked with glutaraldehyde the β -galactosidase loading test revealed diffusion control above 6 U/cm². An apparent activity of 1 U per cm² membrane area has been determined by measuring the rate of hydrolytic glucose formation from lactose in a double-measuring cell. The sensor had a functional stability between 4 and 30 days depending on the microbial source of the enzyme (Fig. 79).

With a similar lactose sensor, Pilloton et al. (1987) assayed lactose in milk samples and obtained a good correlation with the reduction method described by Fehling. The enzyme was fixed to a nylon membrane. A cellulose acetate membrane (molecular cutoff 100 Dalton) was included in order to eliminate electrochemical interferences. The lifetime of the sensor was 1 month.

Matsumoto et al. (1985) developed a sensor with the same enzyme sequence by immobilizing the enzymes covalently to silanized glassy carbon by glutaraldehyde. The sensor had a half-life of 7 weeks. Electrochemical interferences were compensated for by use of an additional, enzyme-free electrode.

A bienzyme electrode comprising GOD and myrosinase coimmobilized on platinized carbon paper for the determination of the glycosinolates progvitrin and sinigrin has been described by Koshy et al. (1988). The substrates, otherwise known as mustard oil glycosides, were deter-

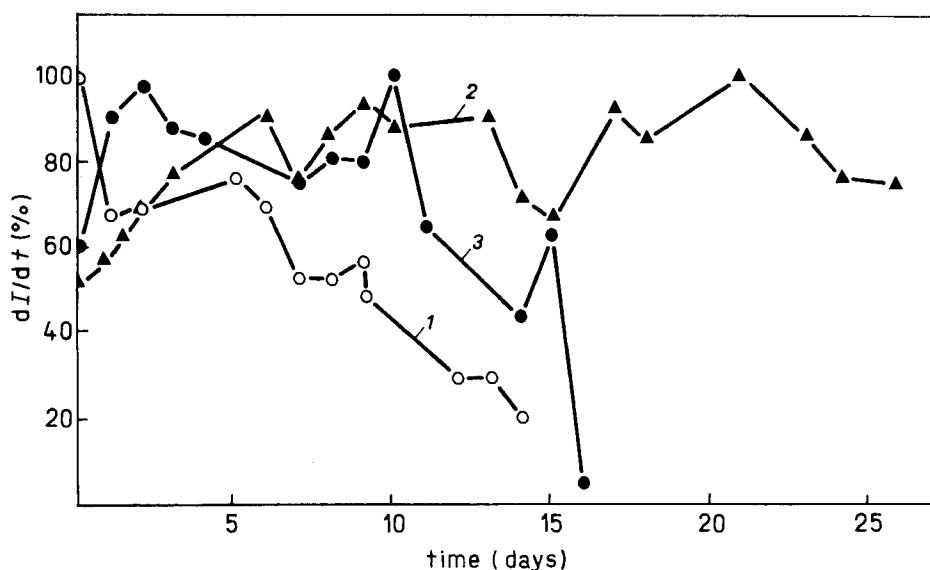


Fig. 79. Lifetime of lactose electrodes using β -galactosidase from *Bifidobacterium* (1), *Escherichia coli* (crosslinked) (2), and *Curvularia inaequalis* (3).

mined in rape seed extracts with acceptable precision. The linear measuring range of the sensor extended from 0.5 to 5 mmol/l. In this range the sensitivity for glucosinolates was about 40% of the respective glucose signal of the bienzyme electrode. Contributions by interfering substances present in the extracts were eliminated by subtracting the response of an analogous GOD electrode from the glucosinolate sensor signals.

The concept of sequentially acting enzymes can also be achieved by combination of several enzyme reactors with one another or with enzyme electrodes in flow systems. Scheller et al. (1987c) proposed an FIA manifold for the determination of maltose and starch containing a glucoamylase reactor upstream of a GOD flow-through electrode (Fig. 80). Starch or maltose are hydrolyzed in the reactor and the liberated glucose is measured at the electrode. When large sample volumes were injected, the steady state signals for both maltose and Zulkowski-starch were constant for reactor residence times between 24 and 73 s. They decreased upon addition of mutarotase into the stream, by about 10% with maltose and by about 20% with starch, since the excess of β -glucose was removed. The steady state value for glucose in the presence of mutarotase coincided with that for equivalent amounts

of both maltose and starch indicating a 100% substrate conversion in the glucoamylase reactor. Upon injection of small sample volumes (40 μl) the peak currents obtained were linearly related to the concentration of glucose, maltose, and starch over three decades (Fig. 81). The highest sensitivity was obtained for maltose, i.e., the lower diffusion coefficient of maltose does not seem to compensate for the excess of β -D-glucose. On the other hand, with starch the effect of diffusion hindrance in the enzyme reactor clearly reduced the sensitivity. The sensor system has been shown to be applicable for the precise determination of up to 30 samples per hour.

Olsson et al. (1986a) described an FIA system comprising several enzyme reactors for sucrose determination in glucose-containing samples. The endogenous glucose was eliminated in a multienzyme reactor filled with immobilized mutarotase, GOD, and catalase. On passing through this reactor, sucrose was converted in another reactor which contained the invertase-mutarotase-GOD sequence. The H_2O_2 formed was measured via a chromogenic reaction conducted in a horse-radish peroxidase reactor. With this complex system a sample throughput of 80/h could be achieved and the interfering glucose signal could be

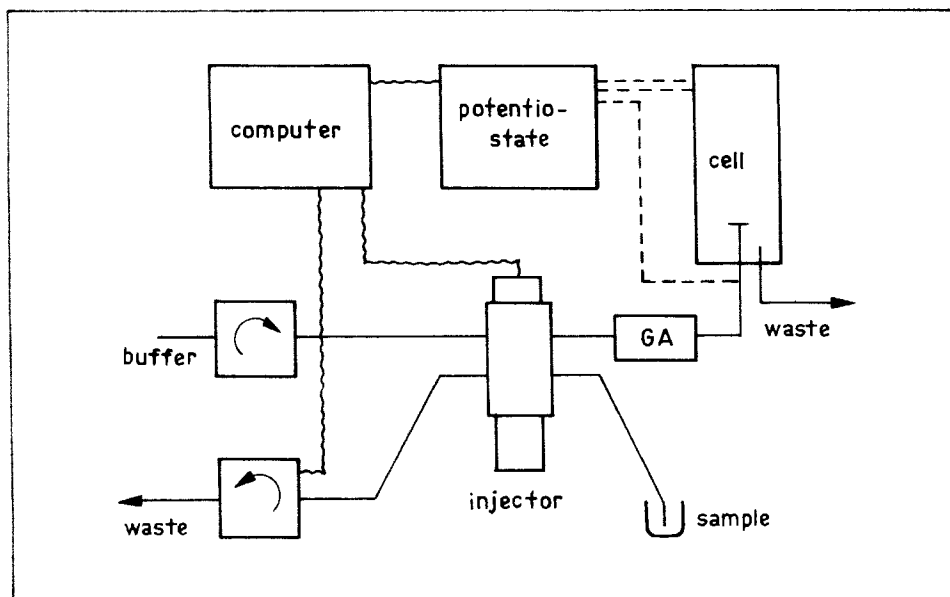


Fig. 80. FIA manifold comprising a glucoamylase (GA) reactor and a GOD flow-through electrode for measurement of maltose and starch. (Redrawn from Scheller et al., 1987c).

suppressed to 0.7% of the sucrose signal. As with most FIA devices the precision was excellent (CV for sucrose = 0.3%).

3.2.1.2 Glucose Oxidase–Peroxidase (–Catalase) and Glucose Oxidase–Gluconolactonase Sensors

In combinations of GOD and HRP, hydrogen peroxide liberated in the glucose oxidation is utilized to oxidize HRP substrates. This sequence is advantageous for glucose assay because the electrochemical indicator reaction can be carried out at much lower potentials than the anodic H_2O_2 oxidation. This is of particular importance with carbon electrodes, since the limiting current of hydrogen peroxide oxidation is above +0.9 V whereas the reduction of ferricyanide occurs already at 0 mV vs SCE. A sensor based on this system has been proposed by Kulys et al. (1983). However, as the direct coupling of the GOD reaction with artificial electron acceptors, such as ferrocene, provides the same advantage and,

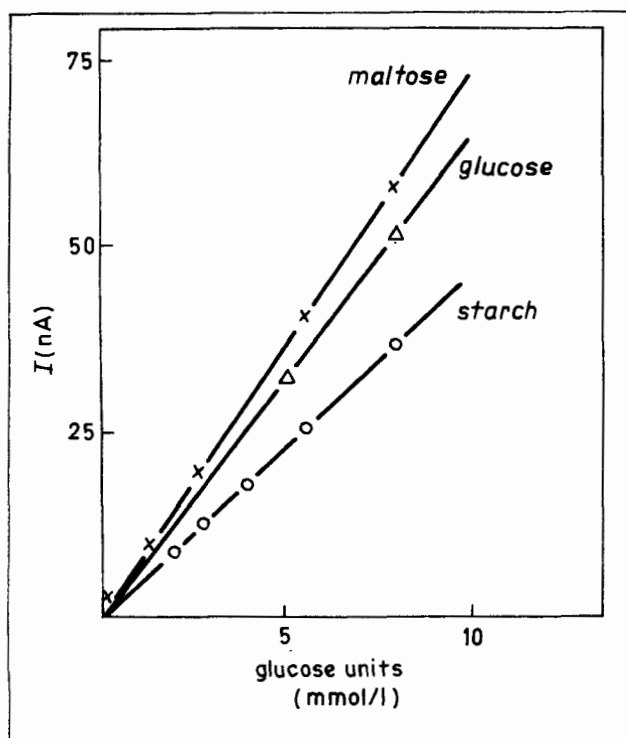


Fig. 81. Concentration dependence of the peak current for starch, maltose, and glucose measured by the equipment shown in Fig. 80.

furthermore, avoids the use of the generally unstable HRP substrates, the sensor has not reached the stage of practical application.

GOD adsorbed on coal electrodes catalyzes the cathodic reduction of hydrogen peroxide in the region of the reversible redox potential (Tarasevich, 1985). The electron exchange is likely to occur directly between the prosthetic group and the electrode. The cathodic limiting current is reached at about 200 mV vs SCE. This decrease of the overpotential appears to be useful for interference-free peroxide detection. Hintsche and Scheller (1987) succeeded in coadsorbing GOD and HRP on carbon electrodes. Hydroquinone served as substrate for HRP. Current measurement at -200 mV vs SCE resulted in a sensitivity 50 times higher than that of a GOD monoenzyme electrode. The higher signal indicates the effect of the electrocatalytic peroxide reduction mediated by HRP.

GOD can also be used for the production of the hydrogen peroxide necessary for the oxidation of the substance to be measured. This principle has been employed to assay the product of heme degradation, bilirubin, by using a GOD-HRP sequence sensor (Renneberg et al., 1982; Scheller et al., 1983b). The measuring solution contains an amount of glucose that is sufficient for complete reduction of the oxygen present in the enzyme membrane to H_2O_2 (Fig. 82). Therefore, variations of the glucose concentration in the measuring cell resulting from sample addition do not cause changes of the hydrogen peroxide signal. When the sample contains the HRP substrate, bilirubin, a part of the H_2O_2 is consumed through substrate oxidation and the measuring signal

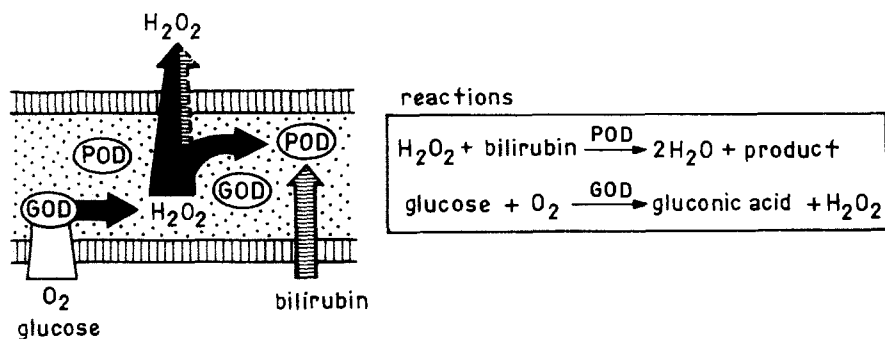


Fig. 82. Schematic representation of bilirubin assay by using a GOD-HRP sequence electrode. (Redrawn from Renneberg et al., 1982).

decreases accordingly. The sensitivity of this sensor was not high enough for determination of bilirubin in serum.

The above measuring principle has been modified for the measurement of HRP substrates which are themselves electrochemically active. To avoid electrochemical interference a Clark-type oxygen probe was used together with catalase coimmobilized with GOD. The hydrogen peroxide not consumed by HRP is cleaved by catalase to oxygen which is indicated at the electrode. Consequently, the biochemical basis of the sensor is the competition of HRP and catalase for the common substrate, H_2O_2 .

The combination of GOD with catalase in potentiometric glucose sensors has been discussed in Section 3.1.1.2. The same enzyme system has also been employed in amperometric enzyme electrodes based on oxygen probes. The sensitivity of glucose measurement is diminished by the action of catalase but the cleavage of H_2O_2 has been shown to enhance the stability of GOD (Buchholz and Gödelmann, 1978). In contrast, Kirstein et al. (1980) found that other oxygen species are responsible for the inactivation of reduced GOD. Furthermore, the activity decay of catalase is usually faster than that of GOD and thus affects the sensitivity of the sensor for glucose. Catalase–GOD electrodes have not, therefore, found general acceptance.

In enzyme thermistors for glucose the use of coimmobilized catalase more than doubles the sensitivity because the reaction enthalpy of hydrogen peroxide cleavage, 100.4 kJ/mol, exceeds that of glucose oxidation, 80.0 kJ/mol. Besides, the regeneration of oxygen expands the linear measuring range. These advantages have promoted the employment of the oxidase–catalase sequence also for the determination of other analytes (Danielsson, 1982).

Hanazato et al. (1988) demonstrated the crucial importance of the rate of gluconolactone hydrolysis in pH sensitive glucose sensors. The authors coimmobilized GOD and gluconolactonase (EC 3.1.1.17) in a 1 μm thick film on the gate region of a pH-FET. The buffer concentration used for glucose measurement was 10 mmol/l. In the absence of lactonase up to 2.5 mmol/l glucose no pH response was found. Optimal sensitivity for glucose was obtained when the lactonase activity of the two-enzyme combination was at least twice as high as the GOD activity.

3.2.1.3 Glucose Isomerase–Glucose Oxidase Sensor

Glucose isomerase accelerates the equilibrium formation between α -D-glucose and D-fructose. The enzyme reaction requires Co^{2+} or Zn^{2+}

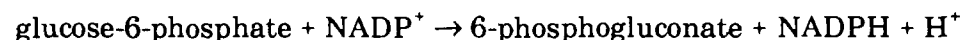
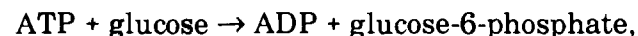
ions. The pH optimum of the reaction is above pH 8, and the temperature optimum above 80°C.

The coupled reactions of isomerase, mutarotase and GOD should be applicable to assay fructose, but a sensor using this sequence has not been described to date. An FIA manifold containing an enzyme reactor with glucose isomerase and mutarotase for the determination of fructose has been developed by Olsson (1987). The glucose formed was measured spectrophotometrically.

Gondo et al. (1981) tried the employment of glucose isomerase in a GOD sensor to remove α -D-glucose. A crude preparation of glucose isomerase was immobilized together with GOD or in a separate membrane, and the membranes were attached to the electrode. The sensitivity and lifetime of the sensor were substantially higher than those of a simple GOD sensor of the same initial activity. The authors concluded that GOD is inhibited by α -D-glucose. This conclusion appears to be doubtful, since the isomerase employed was not shown to be active in the presence of the required activators.

3.2.1.4 Sequence Electrodes for ATP and Glucose-6-Phosphate

The combination of the reactions of hexokinase and glucose-6-phosphate dehydrogenase (G6P-DH):



is well established in analytical enzymology. Its application in a biosensor, as shown in Fig. 83, offers several analytical possibilities (Schubert et al., 1986a). The reaction sequence is coupled to an electrode via oxidation of NADPH by NMP^+ and measurement of the reoxidation by oxygen of the formed NMPH_2 at an oxygen probe. Each substrate and cofactor of the two enzymes then gives a measuring signal of different magnitude (Fig. 84). These different sensitivities are due to the interplay of diffusion and enzyme reaction and, for NADP^+ , to the chemical regeneration by NMP^+ . Since fructose is also phosphorylated in the presence of hexokinase, the sensor is suitable to detect fructose as well. This assay is based on substrate competition, the principle of which will be presented in Section 3.2.2.

Alternatively, glucose-6-phosphate can be determined by using a sequence of alkaline phosphatase (EC 3.1.3.1) and GOD. Such a sequence electrode has been used for the measurement of the competitive inhibitor of phosphatase, inorganic phosphate (see Section 4.4).

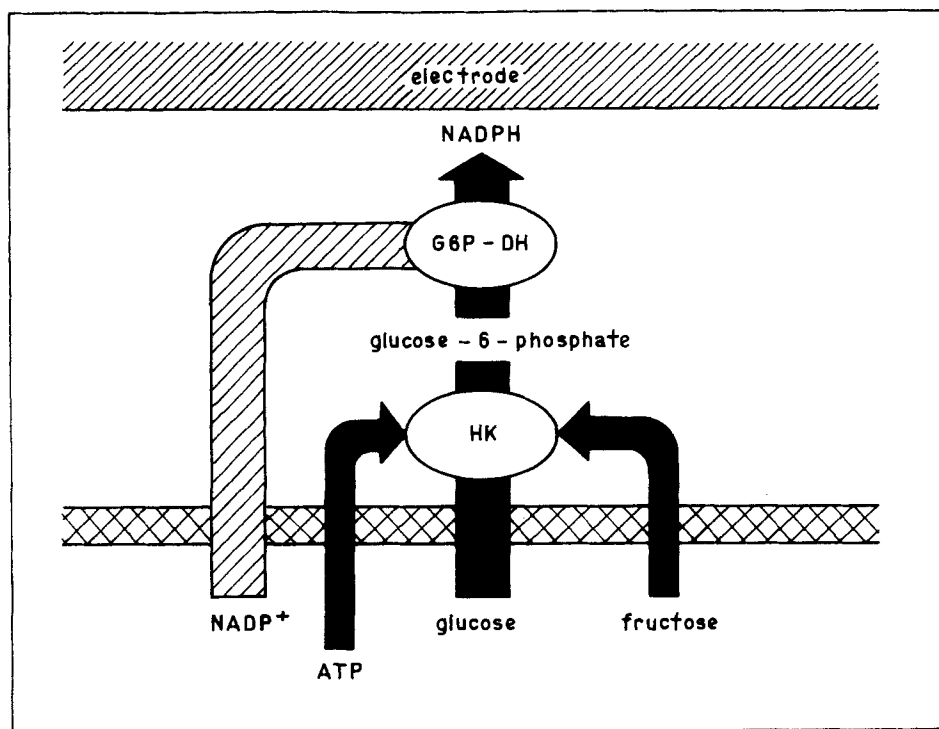


Fig. 83. Principle of a sensor containing hexokinase (HK) and glucose-6-phosphate dehydrogenase (G6P-DH) for determination of NADP^+ , ATP, glucose-6-phosphate, and fructose.

3.2.1.5 Enzyme Sequences Converting Lactate and Pyruvate

With a constant of $K = 2.76 \cdot 10^{-5} \text{ mol/l}$ (pH 7.0, 25°C) the equilibrium of the LDH-catalyzed reaction lies far to the lactate side. This means that whereas for lactate sensors based on LDH the forward reaction has to be forced by alkaline buffer and pyruvate- or NADH-trapping agents, the reduction of pyruvate proceeds spontaneously under normal conditions. This direction of the reaction has been used in a sequence electrode for pyruvate assay (Weigelt et al., 1987b). In the presence of lactate monooxygenase (LMO) lactate formed from pyruvate by LDH is oxidized by molecular oxygen, the consumption of which was indicated at a Clark-type electrode. The enzymes were immobilized in a gelatin membrane. Of course such a sensor measures the concentration of lactate in the sample, too. Therefore it is suited to the determination of the lactate/pyruvate ratio, which is a clinically important parameter. Pro-

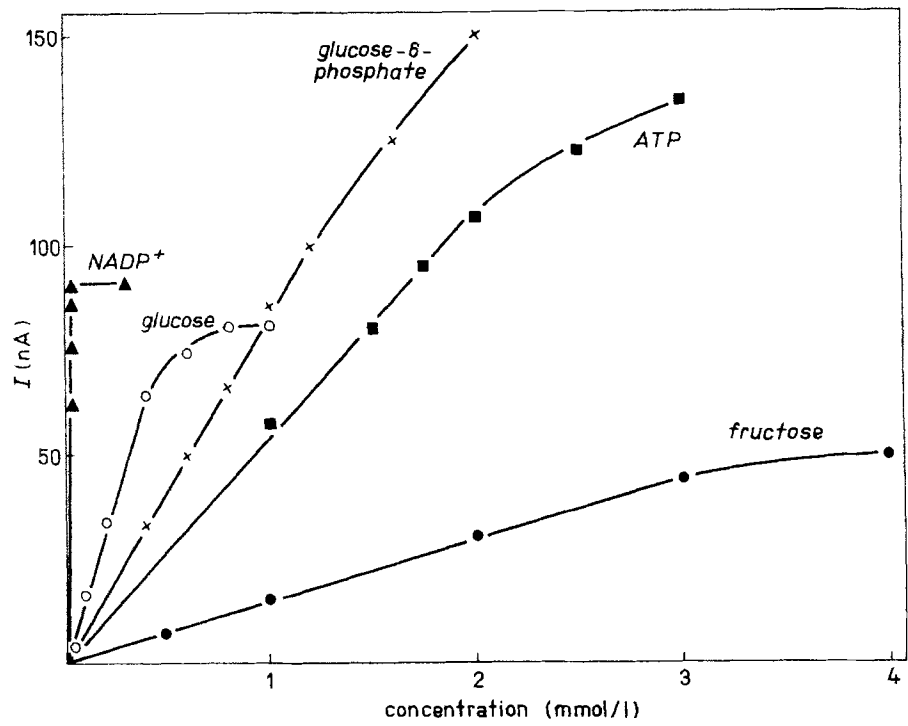


Fig. 84. Dependence of the current signal of the sensor depicted in Fig. 83 on the concentrations of the substrates and cofactors.

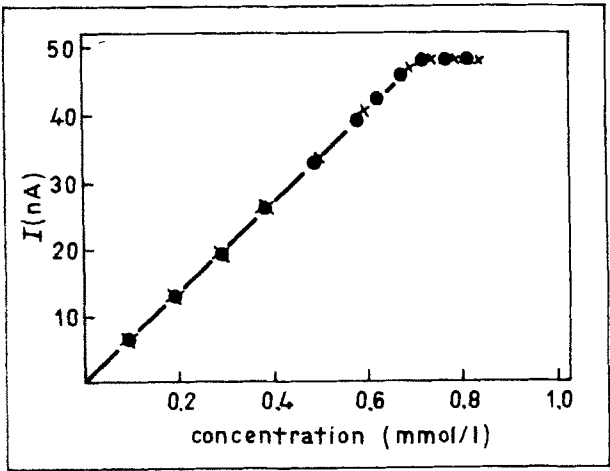


Fig. 85. Calibration curves of an LDH-LMO sensor for lactate (●) and pyruvate (x). (Redrawn from Weigelt et al., 1987b).

vided enough enzyme is immobilized to achieve diffusion control, the coincidence of the diffusion coefficients of lactate and pyruvate makes the sensor identically sensitive to both substrates (Fig. 85). This property is unusual for enzyme sequence sensors and facilitates the calibration of the electrode by reducing it to only one substrate.

The lactate oxidation catalyzed by LMO forms the basis of several other multienzyme electrodes (Fig. 86). The LDH-LMO sensor has also been used to assay the activity of alanine aminotransferase (ALAT, EC 2.6.1.2) and pyruvate kinase (PK, EC 2.7.1.40) (Weigelt 1987; Weigelt et al., 1988). The sample was added to the NADH-containing measuring solution and when the steady state signal for endogenous lactate and pyruvate was attained the substrates of the enzyme to be determined

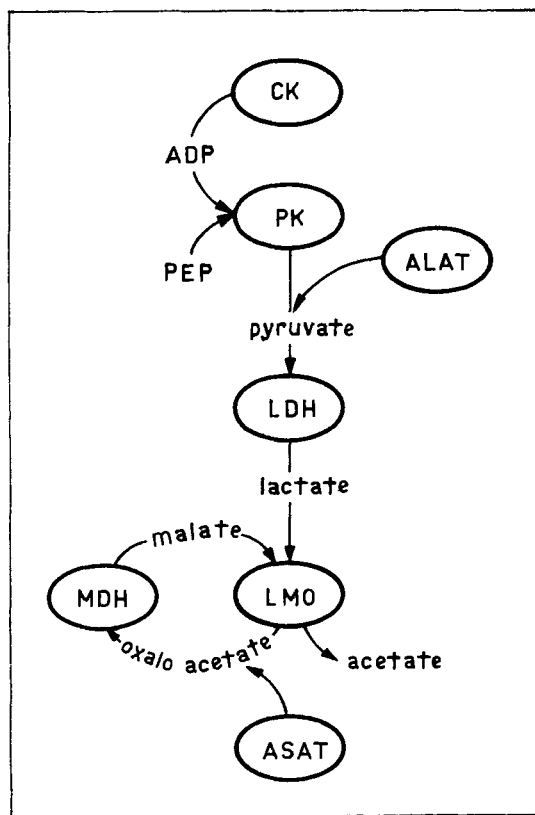


Fig. 86. Coupling of enzyme reactions for the design of a sensor family based on LMO. CK = creatine kinase, PK = pyruvate kinase, MDH = malate dehydrogenase, PEP = phosphoenolpyruvate, ALAT = alanine aminotransferase, ASAT = aspartate aminotransferase.

were added. The slope of the subsequent current decrease resulting from pyruvate formation was linearly related to the activity of ALAT between 0.1 and 90 U/l and to the activity of PK between 5 and 110 U/l. A sensor for the measurement of creatine kinase, lactate and pyruvate was assembled by coimmobilization of PK with LDH and LMO (Fig. 87) (Weigelt et al., 1988).

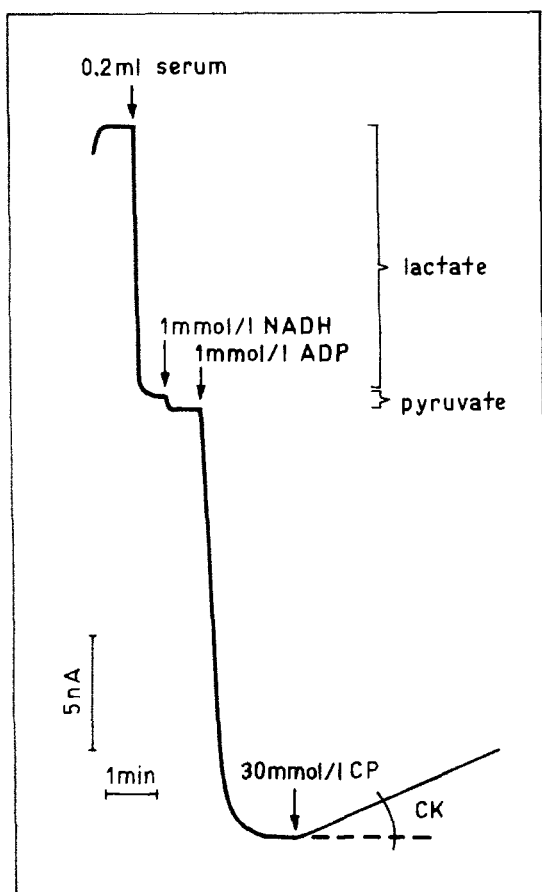
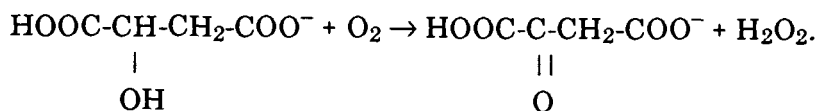
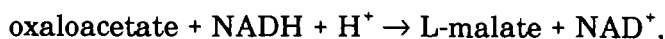


Fig. 87. Response curve of the sequential determination of lactate, pyruvate, and creatine kinase activity with a LMO-LDH-PK sensor. CP = creatine phosphate, lactate: 3.4 mmol/l, pyruvate: 245 μ mol/l, CK: 504 U/l.

Apart from catalyzing the (physiological) oxidative decarboxylation of lactate, LMO is also capable of oxidizing L-malate:



Using this reaction, Weigelt (1987) and Schubert et al. (1991) employed the LDH-LMO sensor for assaying ALAT and aspartate aminotransferase (ASAT, EC 2.6.1.1). For this purpose, malate dehydrogenase (MDH, EC 1.1.1.37) was additionally immobilized in the enzyme membrane. ALAT was determined as described above, and the ASAT reaction then initiated by substrate injection. MDH reduces the oxaloacetate formed to malate:



which was indicated by the LMO-catalyzed oxidation with oxygen. The oxaloacetate regenerated during oxidation is again available for MDH and is thus continuously recycled between LMO and MDH. The recycling compensates for the low sensitivity for malate, which is due to the low affinity of LMO for this intermediate, so that both transaminase products can be measured with nearly the same sensitivity (Fig. 88).

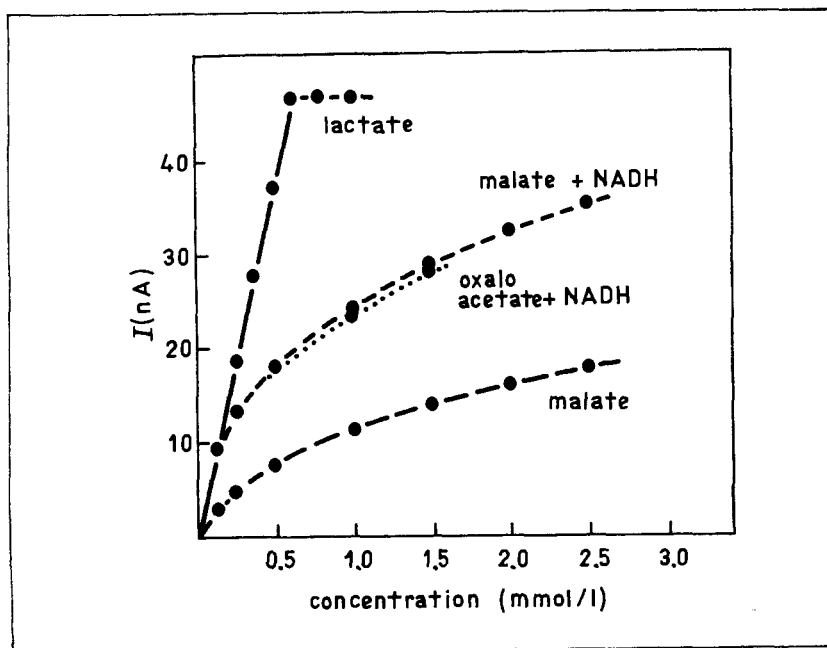


Fig. 88. Calibration graph of the LMO-LDH electrode for lactate, oxaloacetate and malate.

The successive determination of both transaminases has also been carried out with a sequence electrode containing oxaloacetate decarboxylase and pyruvate oxidase (Kihara et al., 1984b). The enzymes were coadsorbed on a PVC membrane of 40 μm thickness in the presence of thiamine pyrophosphate, FAD, and MgCl_2 .

3.2.1.6 Cholesterol Oxidase–Cholesterol Esterase Sequence Sensors

Since in biological material, such as blood serum, cholesterol is partially esterified with fatty acids, a hydrolytic reaction is required for the assay of total cholesterol. This can be carried out chemically, mostly under alkaline conditions (Richmond, 1973; Kumar and Christian, 1977), or enzymatically (Noma and Nakayama, 1976; Clark, 1977; Coulet and Blum, 1983).

Cholesterol esterase (CEH, EC 3.1.1.13) catalyzes the hydrolysis of many cholesterol esters, ranging from the acetate to the stearate, liberating cholesterol and the corresponding fatty acid. Different temperature optima have been found for CEH, 25°C (Lynn et al., 1982), above 60°C (Clark et al., 1978), and 35°C (Tabata et al., 1981). The pH optimum has been determined to be between 6 and 8 in phosphate buffer of 0.3–1.0 mol/l (Dietschy et al., 1976). Maximum activity has been observed in the presence of 0.05–0.3% Triton X-100. Above 0.3% the enzyme is almost completely inhibited.

Enzyme Reactors

Application of coimmobilized cholesterol oxidase (COD) and CEH provides an economically favorable assay of total cholesterol in blood serum. Tabata et al. (1981) and Rigin (1978) have shown that COD and CEH can be covalently bound to alkylamine glass and porous quartz particles, respectively. The preparations were employed in flow-through devices and the hydrogen peroxide formed was detected colorimetrically and by chemiluminescence measurement. With the former immobilize, total cholesterol up to 10 mmol/l could be assayed with a sample throughput of 50/h and satisfactory precision. Once prepared the reactor was suitable for 1000 measurements within 1 month. The enzymes used in a lumometer permitted the sequential assay of free and total cholesterol in plasma samples in the concentration range 0.1 $\mu\text{mol/l}$ –0.3 mmol/l with a measuring time of 15–20 min.

The coimmobilized enzymes have also been used for total cholesterol determination in serum by using an enzyme stirrer (Huang et al., 1977) and a reactor electrode (Karube et al., 1982b; Yao and Wasa, 1988b). In

these systems the cholesterol concentration was determined from the anodic oxidation current of hydrogen peroxide and the cathodic reduction of ferricyanide at an HRP-modified electrode, respectively. With the reactor electrode developed by Karube et al., serum samples containing between 2.5 and 10 mmol/l total cholesterol could be analyzed with a measuring time of 5 min. In contrast, the enzyme stirrer permits only 5 samples per hour to be processed. The results obtained by using the enzyme stirrer correlated well with those of the photometric method ($r = 0.992$). Several hundreds of assays could be performed; the lifetime of the enzyme stirrer was limited by inactivation of the immobilized CEH.

Enzyme Sequence Electrodes for Cholesterol

Enzyme electrodes comprising coimmobilized COD and CEH can be used for the determination of total cholesterol. On the other hand, coupling of COD with HRP enables cholesterol measurement at low electrode overvoltage, which avoids electrochemical interferences. Fig. 89 shows the diversity of the potential sequences of enzymatic and electrochemical reactions in cholesterol electrodes.

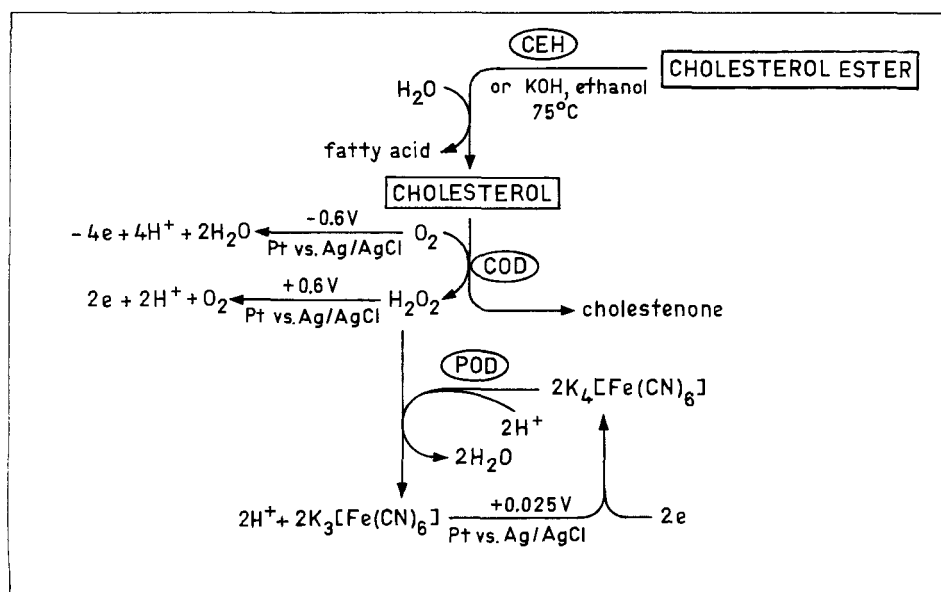


Fig. 89. Coupling of enzymatic reactions with electrochemical detection in amperometric enzyme electrodes for cholesterol. (Redrawn from Wollenberger et al., 1983).

1. Coupling of COD and CEH

Wollenberger et al. (1983) combined COD and CEH immobilized on Spheron particles in a sensor for total cholesterol. The sensor showed no response when separately fixed enzymes were used, but was active with coimmobilized enzymes. In terms of bound activity CEH was 6 times less active than COD. The dependences of the current signal of the enzyme sequence electrode for free and esterified cholesterol were equal for aqueous standard solutions and serum samples (Fig. 90). This indicates a sufficient CEH activity in the immobilized preparation.

The response time under steady state measurement conditions was 5 min, which was double that of a COD monoenzyme electrode (see also Section 3.1.8). The upper limit of the linear current–concentration dependence was 100 $\mu\text{mol/l}$. Owing to the low initial activity of CEH and its rapid inactivation after immobilization the sensor was stable for only 1 day. This is insufficient for practical application.

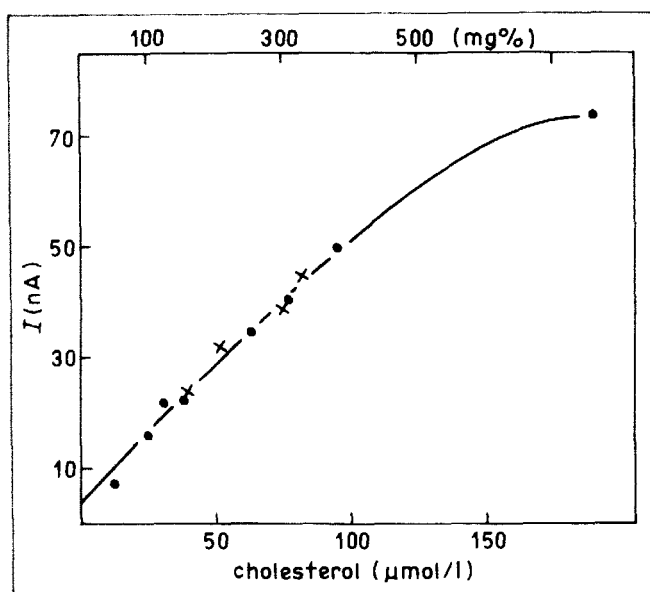


Fig. 90. Calibration curve of an enzyme electrode for the determination of total cholesterol. ●: cholesterol standard solution containing 10% Triton X-100, ×: control serum containing free and esterified cholesterol. (Redrawn from Wollenberger et al., 1983).

2. Coupling of COD and HRP

H_2O_2 formed in the COD reaction can be reduced in an HRP-catalyzed reaction with oxidation of a mediator such as ferrocyanide (Wollenberger

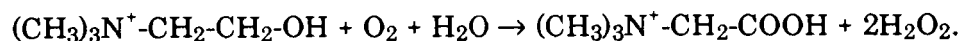
et al., 1983). For this purpose, HRP was immobilized in a gelatin membrane and attached to a platinum electrode covered by a cellulose membrane and polarized at +25 mV vs Ag/AgCl. The enzyme layer was covered with Spheron particles bearing immobilized COD. The carrier was held in place by a silk screen and an O-ring. The electroreduction of ferricyanide formed in the reaction sequence served as the measuring signal. The sensor had a response time of 1–2 min and a lifetime of 2 days.

In a similar manner, but with no necessity to add an electron acceptor, an assay of cholesterol has been designed using an organic metal electrode of $\text{NMP}^+\text{TCNQ}^-$ (Wollenberger, 1984). HRP was adsorbed on this material and covered by nylon-bound COD. The electrode was polarized at +25 mV to obtain a signal upon addition of cholesterol. The working stability of this sensor was poor.

3.2.1.7 Enzyme Sequence Sensors for Phosphatidylcholines and Acetylcholine

Phosphatidylcholines, above all lecithin, occur in millimolar concentrations in serum and bile. Acetylcholine (ACh) occurs in brain and nerve extracts together with its degradation product, choline.

A highly specific assay of choline can be carried out by using choline oxidase (EC 1.1.3.17) catalyzing the reaction:



Choline oxidase has been immobilized, using a variety of procedures, on hydrophobic agarose particles (Karube et al., 1979c), asymmetric acetylcellulose membranes (Mizutani and Tsuda, 1982), and nylon netting (Mascini and Moscone, 1986). Yao (1983) constructed a CME by direct fixation of choline oxidase on a silanized platinum net electrode via glutaraldehyde coupling.

As the choline concentrations to be determined in biological fluids are very low, electrochemical interferences have to be eliminated. For this purpose, Mascini and Moscone (1986) attached a cellulose acetate membrane immediately to the hydrogen peroxide-indicating electrode. Yao (1983) proposed the introduction of a column incorporating a Cu^{2+} -complex for chemical oxidation of interfering species in the sample stream. These methods were suitable for determination of choline concentrations as low as 0.5 $\mu\text{mol/l}$. The sensitivity for choline was about twice as high as that for H_2O_2 . This confirms the formation of 2 moles H_2O_2 per

mole choline and indicates a low diffusion resistance of the enzyme membrane. Aliphatic mono- and diamines were measured with only 2–6% of the sensitivity for choline. The sensors have been used to assay phosphatidylcholine and cholinesterase activity.

For measurement of phosphatidylcholine in serum the samples were preincubated with 0.2–0.5 U phospholipase D (EC 3.1.4.4) in the presence of Triton X-100 and calcium chloride (Mascini et al. 1986; Campanella et al., 1988). The liberated choline was assayed by means of the choline oxidase sensor. To assay lecithin in foodstuff the samples were extracted with ethanol and diluted with buffer. In the LCA 400 Lipid Analyzer of Toyo Jozo (Japan) the same principle is used for serum samples. However, this device appears not to be suitable for routine application and has been withdrawn from the market.

Coimmobilized phospholipase D and choline oxidase have been employed in an enzyme reactor with electrochemical hydrogen peroxide detection (Karube et al., 1979c). This configuration was stable for 9 days.

Yao (1983) has shown that determination of acetylcholine esterase (AChE) activity is feasible with the above mentioned CME. ACh was hydrolyzed in a reaction vessel arranged in an FIA manifold upstream of the choline electrode. With a sample throughput of 40/h the sensor responded linearly to the enzyme activity over the range of 0.25 to 100 mU.

During application of the choline oxidase electrode to serum samples, Mascini and Moscone (1986) observed that the sensor also indicated ACh due to adsorption of AChE from the sample on the enzyme membrane. This finding can be explained by the high surface activity of AChE and its high concentration in normal serum, and has been confirmed by Gruss (1989), who investigated the adsorption of serum on carbon electrodes.

The above authors coimmobilized choline oxidase and AChE on a nylon net which was fixed to a hydrogen peroxide probe so that the esterase was adjacent to the solution. The apparent activities were 200–400 mU/cm² for choline oxidase and 50–100 mU/cm² for AChE. The sensitivity of the sequence electrode for ACh was about 90% of that for choline, resulting in a detection limit of 1 µmol/l ACh. The response time was 1–2 min. The parameters of this amperometric sensor surpass those of potentiometric enzyme electrodes for ACh (see Section 3.1.25). Application to brain extract analysis has been announced.

3.2.1.8 Multienzyme Electrodes for Creatinine and Creatine

In contrast to creatinine sensors using the direct indication of creatinine cleavage, amperometric assays require enzyme sequences. Tsuchida and Yoda (1983) and Yoda (1988) immobilized creatinine amidohydrolase (EC 3.5.2.10), creatine amidinohydrolase (EC 3.5.3.3), and sarcosine oxidase (EC 1.5.3.1) together with BSA by glutaraldehyde crosslinking to an asymmetric hydrogen peroxide selective membrane pretreated with γ -aminopropyl triethoxysilane. The coupled enzymes catalyze the conversion of creatinine to formaldehyde and glycine with the formation of H_2O_2 (Fig. 91). The enzyme membrane contained a total of 1.4 mg protein per cm^2 . The immobilization gave rise to an increase in the apparent K_M values of the hydrolases, a decrease in the apparent K_M value of sarcosine oxidase, and a shift of the pH optimum by 2.5 units to the alkaline side. The buffer, which had a compromise pH of 7.5, contained azide, Mg^{2+} -EDTA, and hypophosphite to stabilize and activate the hydrolases. The sensitivity ratio of the trienzyme electrode for creatinine, creatine, and sarcosine was 0.62:0.59:1.

For creatine determination the appropriate bienzyme electrode was used. Creatinine and creatine could be assayed in parallel by combining a trienzyme electrode and a bienzyme electrode. Both electrodes reached a steady signal 2 min after sample addition. With a 25 μl sample volume the linear range was 1–100 mg/l. The sensors were stable for more than 500 measurements. The CV of creatine determination in serum was 1.3% with 21.7 mg/l and 11.7% with 8.2 mg/l, and a day-to-day CV of 8.4% was achieved. Comparison with the Jaffé method for 55 serum

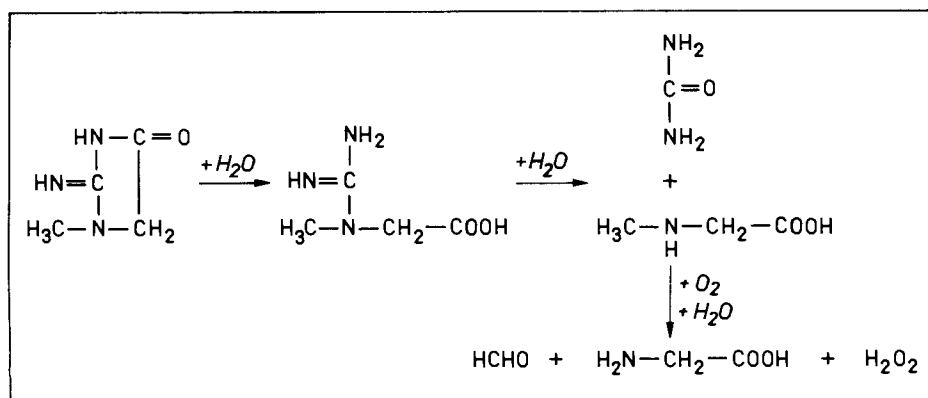


Fig. 91. Successive enzymatic conversion of creatinine to sarcosine.

samples yielded a correlation coefficient of 0.985 with an equation of $y = (1.078x - 2.6)$ mg/l. Using the rate method the total measuring time amounted to 100 s. These sequence sensors are superior to monoenzyme electrodes for creatinine and creatine with respect to sensitivity, measuring time, and analytical quality. The good operational parameters seem to justify the effort required for the preparation of the enzyme sequence sensors.

Dittmer et al. (1988) entrapped the three enzymes in gelatin and obtained a functional stability of 15 days for the measurement of creatinine, creatine and sarcosine. Obviously the mild immobilization procedure leads to diffusion control in the enzyme membrane. After some days of operation the sensitivity for creatinine and creatine gradually decreased while that for sarcosine remained constant.

3.2.1.9 Multienzyme Electrodes for Nucleic Acid Compounds, Phosphate and Fatty Acids

During the storage of meat, nucleic acids are degraded leading to the formation of nucleotides. The concentration of the degradation products is a measure of the freshness of the meat.

Watanabe et al. (1986) developed a sequence sensor for the successive assay of hypoxanthine (HX) and inosine (HXR) by arranging nucleoside phosphorylase (EC 2.4.2.1) and xanthine oxidase (EC 1.2.3.2) in spatially separated layers in front of an oxygen probe. Nucleoside phosphorylase was

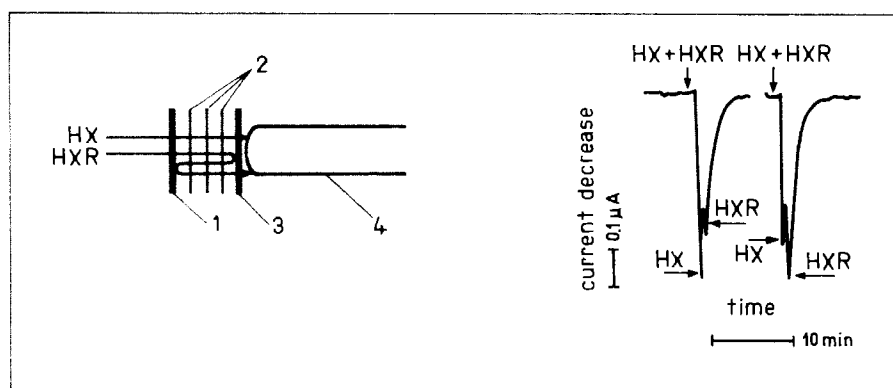


Fig. 92. Schematic and response curve of successive determination of hypoxanthine (HX) and inosine (HXR) with a multilayer electrode. 1: xanthine oxidase, 2: cellulose triacetate membrane, 3: nucleoside phosphorylase, 4: oxygen electrode. (Redrawn from Watanabe et al., 1986).

attached adjacent to the oxygen permeable sensor membrane whereas xanthine oxidase was separated from this layer by four triacetylcellulose membranes (Fig. 92). This arrangement provides the oxidation of HX in the layer contacting the measuring solution. By contrast, inosine has to permeate this layer and the four dialysis membranes before being converted in the nucleoside phosphorylase layer. The reaction product, hypoxanthine, has to diffuse back to the xanthine oxidase layer to be converted there with consumption of oxygen. The differences in the diffusion distances for HX and HXR result in differences in the response behavior permitting the time resolution of the two measuring signals (Fig. 92b). Ten minutes were needed for such a double measurement, the linear measuring range being 0.1–0.4 mmol/l for HX and 1–40 mmol/l for HXR. The sensitivity for HXR was 10% of that for HX. This measuring principle is also intended for application to the assay of mixtures of glucose and disaccharides, and cholesterol and cholesterol esters.

Gotoh et al. (1988) constructed a sensor for hypoxanthine by immobilizing xanthine oxidase on the gate of an amorphous silicon ISFET. The enzyme-FET responded to HX in the range 0.02–0.1 mmol/l. By coimmobilization of nucleoside phosphorylase, inosine could be measured in the same concentration range.

Since inorganic phosphate is indispensable for the nucleoside phosphorylase reaction, the phosphate concentration can be converted into an oxygen signal by using a nucleoside phosphorylase–xanthine oxidase sequence electrode (Watanabe et al., 1987; Watanabe, 1988). 2 mmol/l of inosine has been shown to be necessary for optimal sensitivity to phosphate ion. The measuring range was 0.1–1 mmol/l and the sensor could be used for 70 assays.

A sensor system for the determination of the freshness of meat has been proposed by Watanabe et al. (1984). It consists of a flow-through cell containing four enzyme electrodes which incorporate the following enzymes:

1. xanthine oxidase (XO) for hypoxanthine (HX),
2. XO + nucleoside phosphorylase (NP) for inosine (HXR),
3. XO + NP + 5'-nucleotidase (NT, EC 3.1.3.5) for inosine-5'-phosphate (IMP),
4. XO + NP + NT + 5'-adenylate deaminase (AD, EC 3.5.4.6) for AMP.

The enzymes were covalently bound to acetylcellulose membranes by glutaraldehyde and the membranes attached to oxygen electrodes.

Measurements were conducted in phosphate buffer, pH 7.8, containing 0.1 mmol/l cysteine.

For a given substrate, e.g. hypoxanthine, the sensitivity decreased with increasing number of enzymes, i.e. from the monoenzyme electrode to the four-enzyme electrode. The sensitivity of each enzyme sensor decreased likewise in the order HX, HXR, IMP, AMP. This indicates kinetic control by several enzyme reactions. In order to measure the concentrations of all four of the analytes in a sample, the sensitivity of each sensor for each substrate must be known because the current changes are coupled with each other according to:

$$\Delta I_1 = 0.15 \text{ HX},$$

$$\Delta I_2 = 0.12 \text{ HX} + 0.05 \text{ HXR},$$

$$\Delta I_3 = 0.073 \text{ HX} + 0.032 \text{ HXR} + 0.0086 \text{ IMP},$$

$$\Delta I_4 = 0.076 \text{ HX} + 0.043 \text{ HXR} + 0.0076 \text{ IMP} + 0.004 \text{ AMP}.$$

After calibration and measurement the currents were evaluated using a computer. In this manner, four measurement values from a complex sample could be obtained within 5 min without the performance of any separation step. The AMP sensor has been extended to the recognition of free fatty acids having a 6–10 carbon atom chain by coupling of acyl-CoA synthetase. In the reaction catalyzed by this enzyme acyl-CoA and AMP are liberated from fatty acids in the presence of ATP, CoA and Mg^{2+} . AMP enters the four-enzyme sequence incorporated in the sensor.

3.2.2 Competition Sensors

Two fundamentally different types of competing reactions can be utilized in biosensors: *enzyme competition*, i.e., the competition of different enzymes for one and the same substrate, and *substrate competition*, i.e., the competition of different substrates for a single enzyme. The latter may be regarded as an analogy to the determination of competitive inhibitors (see Section 4.2). Similar to enzyme sequences, these competition systems serve to gain access to analytes, not involved in enzyme reactions, which can be electrochemically monitored. Table 12 gives an overview of enzyme and substrate competition sensors.

The cofactor-dependent competition of two enzymes for glucose is the basis of sensors for the determination of ATP and NAD^+ (Pfeiffer et al., 1980). GOD has been coimmobilized with hexokinase and glucose dehydrogenase (GDH), respectively, and attached to the tip of an oxygen

TABLE 12
Competition Sensors

Reaction type	Analyte	Enzymes	References
Enzyme competition	ATP	GOD + hexokinase	Scheller and Pfeiffer (1980)
	NAD ⁺	GOD + glucose dehydrogenase	Pfeiffer et al. (1980)
	aminopyrine	HRP + catalase	Renneberg et al. (1982)
Substrate competition	fructose	hexokinase + glucose-6-phosphate dehydrogenase	Schubert et al. (1986a)
	fructose	hexokinase + GOD	Schubert and Scheller (1983)
	phenols	hemoglobin	Scheller et al. (1987c)

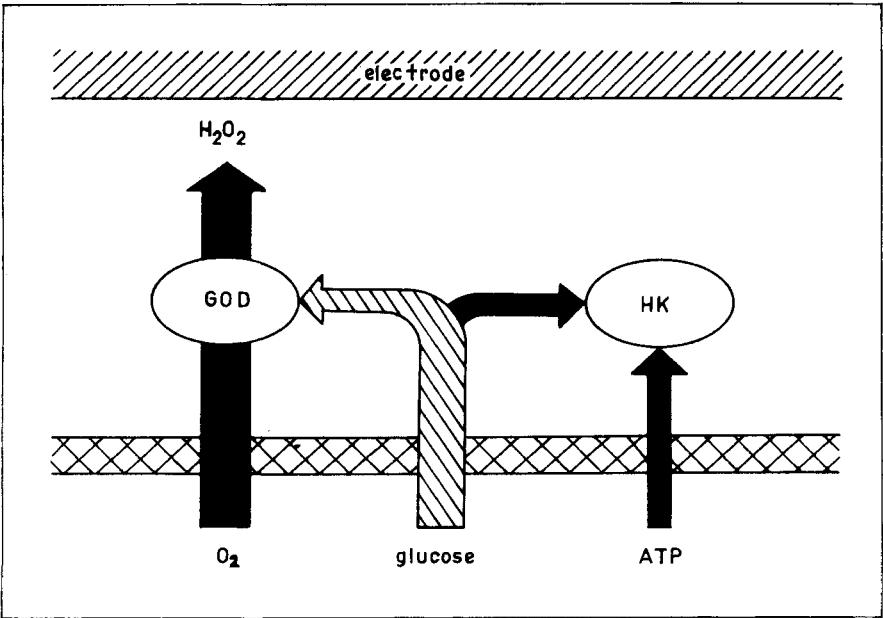


Fig. 93. Glucose oxidase–hexokinase electrode for ATP determination.

electrode (Fig. 93). If glucose is present in the measuring solution it is consumed solely by GOD, giving rise to an oxygen consumption detected

by the electrode. Addition of ATP or NAD^+ starts the relevant second enzyme reaction, which then consumes a part of the glucose, thereby withdrawing it from GOD. The magnitude of this competition is indicated by a diminished oxygen consumption and depends on the cofactor concentration. The sensors exhibited linear current-concentration relations up to 2 mmol/l for ATP and up to 1 mmol/l for NAD^+ . Renneberg et al. (1982) described an enzyme competition sensor for substrate determination based on catalase and horseradish peroxidase. Hydrogen peroxide added to the measuring solution was cleaved by catalase with the formation of oxygen which was sensed at the electrode. In the presence of its substrates, HRP competes with catalase for H_2O_2 and diminishes the oxygen reduction current. Aminopyrine was used as a model substrate for HRP. With the enzymes coimmobilized in one membrane a linear calibration curve up to 1 mmol/l was obtained. When separate membranes were used, one bearing catalase and the other HRP, the sensitivity was enhanced and the linear range narrowed.

Substrate competition has been utilized for fructose assay with a sensor based on immobilized hexokinase and glucose-6-phosphate dehydrogenase as described in Section 3.2.1.4 (Schubert et al., 1986a). The NADPH formed was detected at an O_2 electrode via the mediator NMP^+ . In this complex reaction scheme (see Fig. 83), fructose changes the glucose signal by competing with glucose for hexokinase. In the presence of a constant glucose concentration the sensor responds linearly to fructose between 0.3 and 3 mmol/l (see Fig. 84). This type of competition for hexokinase has also been studied by using a sensor with coimmobilized hexokinase and GOD, which combines enzyme competition and substrate competition (Schubert and Scheller, 1983). Linearity was found between 0.05 and 1 mmol/l fructose.

Scheller et al. (1987c) described a substrate competition electrode for the determination of aniline and phenol (Fig. 94). The analyte competes with hydroquinone for the pseudo-peroxidatic activity of hemoglobin. The decrease of the electrochemical reduction current of benzoquinone in the presence of the alternative substrate served as the measuring signal.

3.2.3 Enzymatic Elimination of Interferences

The applicability of biosensors may be severely restricted by disturbances caused by sample constituents interfering with the binding or enzymatic conversion of the analyte, such as inhibitors or alternative

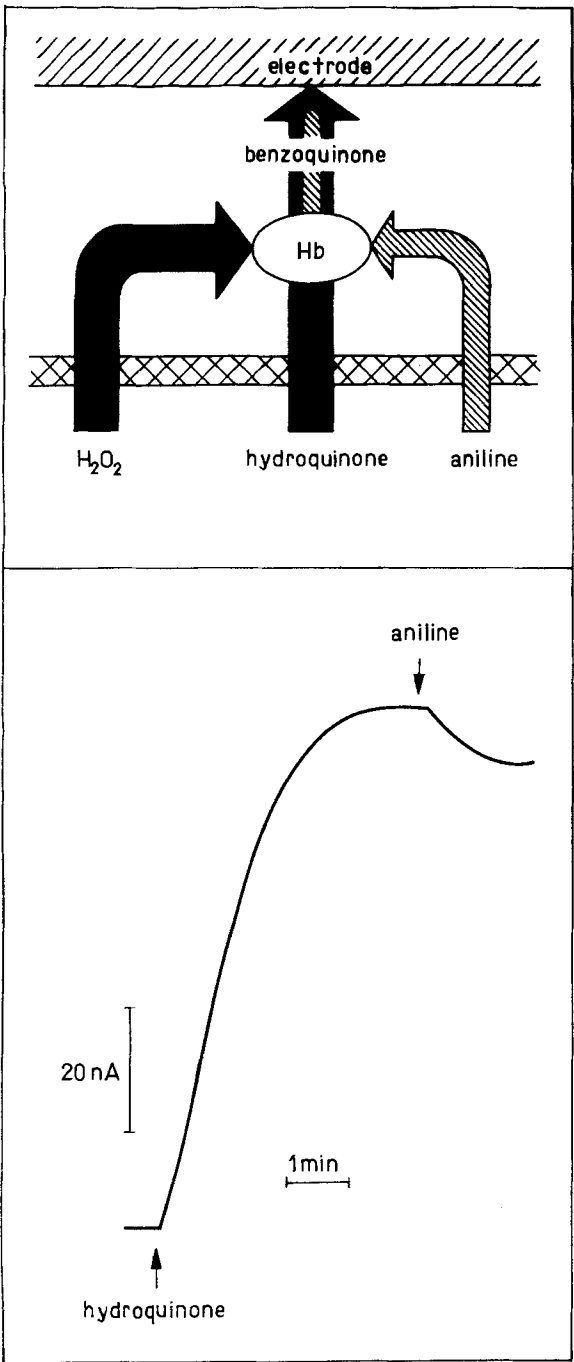


Fig. 94. Schematic and measuring curve of a substrate competition sensor for phenols and aniline using immobilized hemoglobin (Hb). (Redrawn from Scheller et al., 1987c).

substrates. In particular, in biosensors using coupled enzyme reactions the substrates of each reaction will interfere; increasing complexity of biosensors thus results in a decreased selectivity. Interferences can also occur at the level of the transducer reaction.

Such interferences can be compensated by using difference measurements, which usually require a reference transducer. An elegant alternative is the use of *enzymatic anti-interference systems* containing enzymes in front of the sensor that catalyze the conversion of the disturbing compounds to inert products. Such systems have been developed in conjunction with analytical enzyme reactors as well as enzyme electrodes.

Mascini et al. (1985a) employed creatinine iminohydrolase immobilized on nylon tubing for the determination of creatinine in serum. The reaction product, NH_3 , was indicated at an ion selective electrode. To avoid signal superposition by endogenous ammonia the sample was first mixed with α -ketoglutarate and pumped through a tube containing immobilized glutamate dehydrogenase. The tube was capable of removing endogenous NH_3 up to 0.1 mmol/l according to the reaction:



The same reaction system, immobilized on porous glass, has been used by Winquist et al. (1986) in two reactors combined with an ammonia sensitive iridium metal oxide semiconductor structure in an FIA system. NH_3 up to 0.2 mmol/l could be completely removed in the glutamate dehydrogenase reactor.

Olsson et al. (1986a) arranged two reactors in series, one containing mutarotase, GOD, and catalase and the other, for sucrose measurement, containing invertase, mutarotase, and GOD. In the first reactor sample glucose is oxidized to gluconic acid and water and is thus prevented from reaching the reaction sequence for sucrose determination. The anti-interference reactor was capable of removing glucose in the sample up to 1 mmol/l.

Matsumoto et al. (1988) coupled enzyme reactors for the elimination of glucose and ascorbic acid with enzyme reactors for the determination of sucrose, glucose and fructose in an FIA manifold.

In biospecific electrodes the eliminating enzymes are directly integrated in the sensor in membrane-immobilized form and separated from the indicator enzyme layer by a semipermeable membrane. The first enzymatic anti-interference layer was developed to enable the electrochemical determination of catecholamines in brain tissue at a graphite

electrode (Nagy et al., 1982). A layer of ascorbate oxidase was attached to the electrode to provide oxidation of ascorbic acid before it could reach the electrode surface. The catecholamines could easily diffuse through the membrane to the electrode.

Since many enzyme electrodes are based on glucose measurement by GOD after sequential or competitive analyte conversion, endogenous glucose is a prominent interfering compound. To eliminate endogenous glucose an anti-interference layer containing coimmobilized GOD and catalase has been devised (Scheller and Renneberg, 1983; Renneberg et al., 1983b). This layer is impermeable to glucose up to 2 mmol/l. It has been combined with a glucoamylase–GOD membrane and an invertase–GOD membrane, respectively, for interference-free measurement of α -amylase and sucrose in glucose-containing samples.

Should the sample contain large amounts of glucose, the oxygen consumption in the anti-interference layer can result in a lack of oxygen in the indicator enzyme membrane which would tend to reduce the measuring range of the sensor. To avoid this disadvantage, Scheller et al. (1987b) developed an alternative glucose anti-interference layer by using hexokinase (Fig. 95). It requires only ATP as cosubstrate and is also impermeable to glucose up to 2 mmol/l. The membrane has been

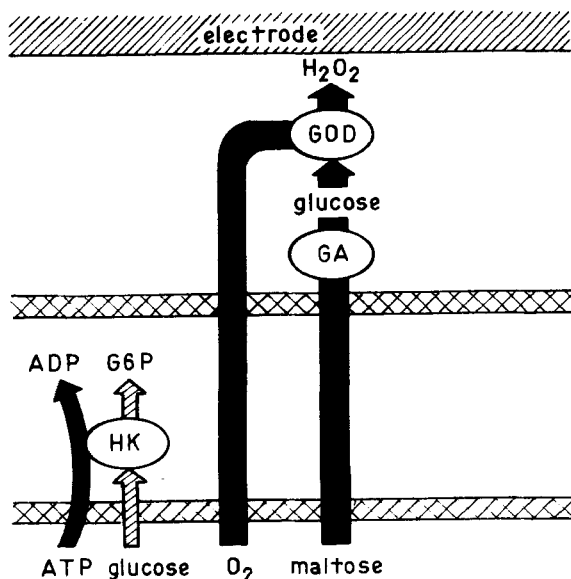


Fig. 95. Enzyme sequence electrode for maltose determination containing a hexokinase anti-interference layer for glucose.

employed in combination with a glucoamylase–GOD membrane for maltose assay in the presence of glucose. It is, furthermore, advantageous in that it permits the use of oxygen-indicating as well as hydrogen peroxide-indicating transducers.

Weigelt et al. (1987a) studied the elimination of lactate by a lactate monooxygenase membrane. An enzyme loading of 10 U/cm^2 was sufficient for complete oxidation of up to 0.6 mmol/l lactate to inert acetate and CO_2 (Fig. 96).

Glucose measurements in urine and fermentation broth by means of GOD electrodes based on hydrogen peroxide detection usually suffer from interference by anodically oxidizable compounds. These can be oxidized in the measuring solution by reaction with hexacyanoferrate(III). In order to prevent the hexacyanoferrate(II) formed from reaching the electrode, Wollenberger et al. (1986) developed an anti-interference membrane incorporating immobilized laccase which reoxidizes the mediator with consumption of oxygen (Fig. 97). The approach

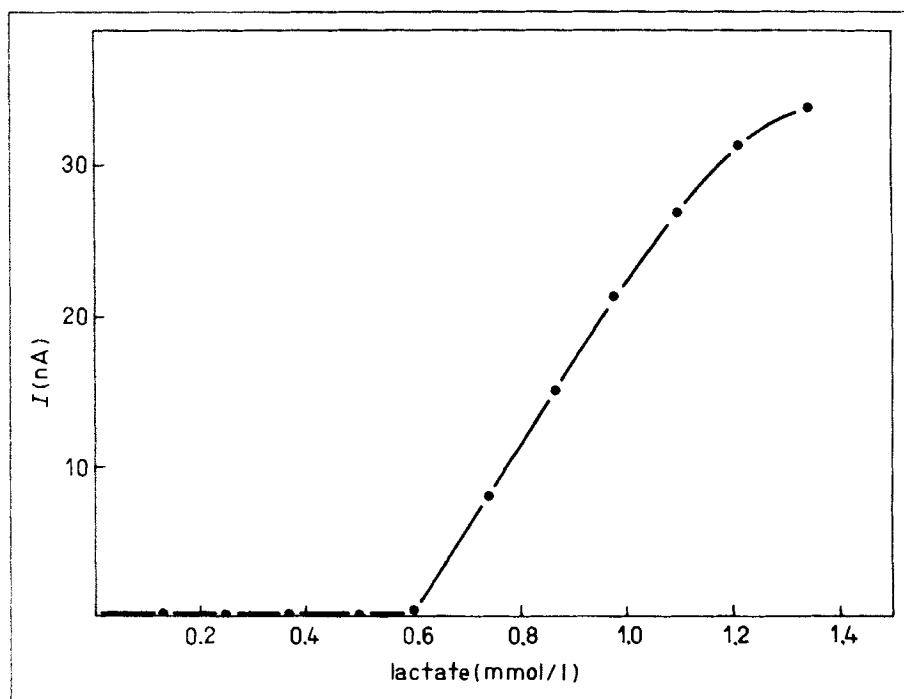


Fig. 96. Function of a lactate monooxygenase membrane as anti-interference layer for lactate. The current is a measure of the permeation of lactate through the membrane.

has been experimentally tested by using ascorbic acid as a model interferent. The system was capable of shielding the electrode from ascorbic acid in concentrations up to 2 mmol/l.

Enzymatic anti-interference layers containing oxidases can also be used to eliminate oxygen or prevent its diffusion into the electrode-near space. This enables the polarographic determination of organic com-

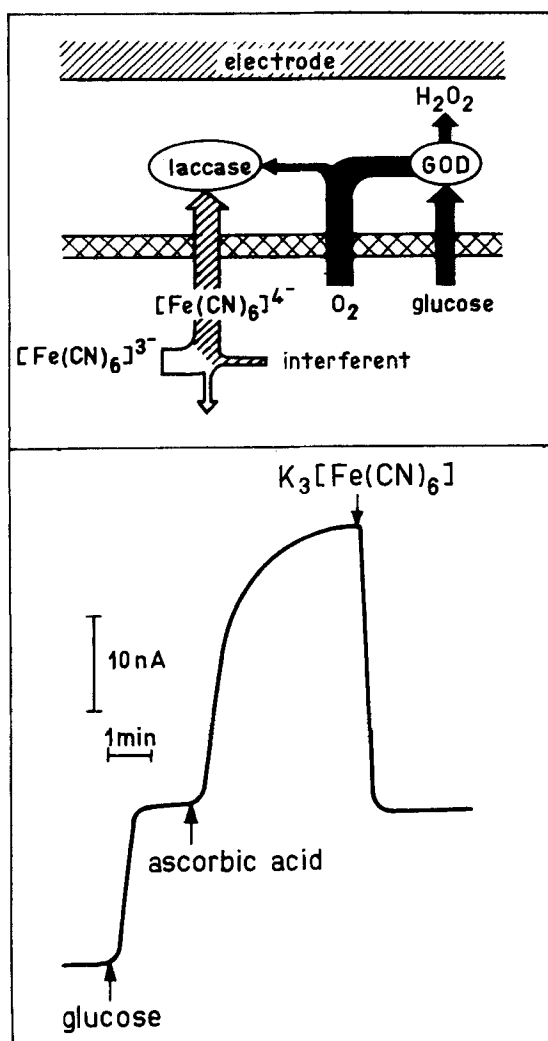


Fig. 97. Principle and measuring curve of a glucose electrode with elimination of interference by the hexacyanoferrate(III)/laccase system. (Redrawn from Wollenberger et al., 1986).

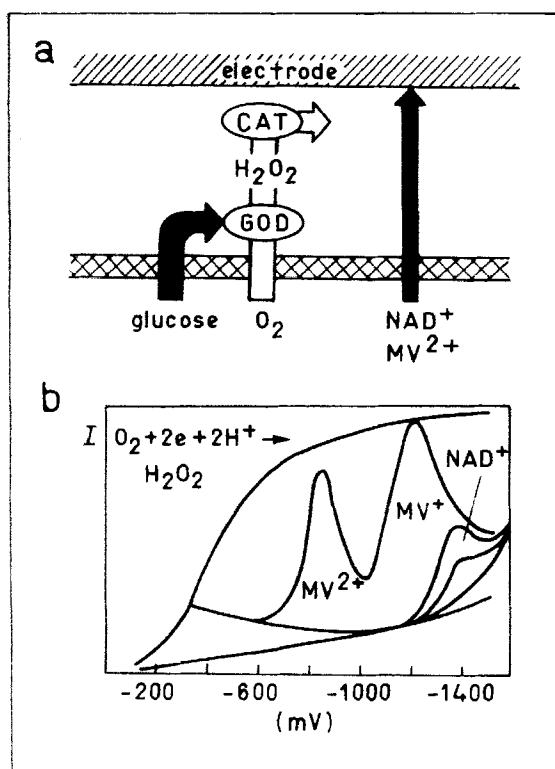


Fig. 98. Differential pulse polarograms (b) of NAD^+ and methylviologen (MV) at an anaerobic flat Hg electrode (a) covered with a GOD-catalase membrane. (Redrawn from Scheller et al., 1987a).

pounds by cathodic reduction without tedious oxygen removal by nitrogen purging or the like. Scheller et al. (1987a) covered a flat mercury electrode with a GOD-catalase membrane and, upon addition of glucose to the measuring solution, observed a drastic decrease of the base current of the electrode at strongly negative potential. Under these conditions, current peaks proportional to the concentration of NAD^+ , pyruvate, and methylviologen were obtained (Fig. 98).

3.2.4 Substrate Recycling

The concentrations of a number of analytically relevant compounds, such as creatinine, pyruvate, hormones, and drugs, are often so low that their assay with enzyme electrodes requires large sample volumes or is totally impossible. The detection limit of usual enzyme electrodes is

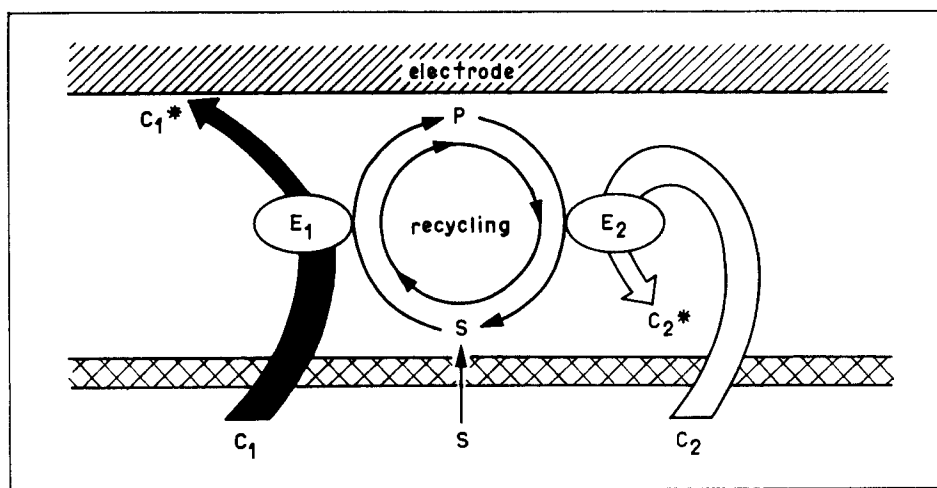


Fig. 99. Signal amplification by enzymatic recycling in enzyme electrodes. E = enzyme, S = substrate, P = product, C = cosubstrate, C* = coproduct.

between 10^{-6} and 10^{-7} mol/l but the physiological concentrations of hormones, growth factors and antibodies are in the nanomolar range or lower.

A substantial enhancement of the sensitivity of enzyme electrodes can be achieved by using substrate recycling. This coupling principle works in an analogous way to the cofactor recycling known from enzymatic analysis with dissolved enzymes. In a bienzyme sensor (Fig. 99) the substrate to be determined is converted by reaction with enzyme 1 to a product which is, in turn, the substrate of enzyme 2. The latter catalyzes the regeneration of the substrate to be determined which thus becomes available for enzyme 1 again, and so forth. One of the coreactants is detectable directly or via an additional reaction. Assuming that enzyme 1 is present in sufficiently high concentration as to assure diffusion control, an amplification is achieved by switching on enzyme 2 by addition of its cosubstrate. In such systems the analyte acts as a catalyst which is shuttled between both enzymes in the overall reaction of both cosubstrates. Consequently, much more cosubstrate will be converted than analyte is present in the enzyme membrane. By this amplification the change of the parameter indicated at the transducer will greatly exceed that obtained with a one-way analyte conversion. The ratio of the sensitivity in the linear measuring range of the amplified and the unamplified regime is termed the *amplification factor*. Table 13 shows the analyte recycling sensors developed to date.

TABLE 13

Substrate Recycling in Biosensors

Analyte	Enzymes	Form of application	Transducer	Amplification factor	References
Lactate	lactate oxidase + lactate dehydrogenase	reactor	thermistor	1000	Scheller et al. (1985a)
		reactor	O ₂ electrode	70	Asouzu et al. (1990)
		membrane	O ₂ electrode	4100	Wollenberger et al. (1987a)
				250	Mizutani et al. (1985)
Glucose	GOD + glucose dehydrogenase	membrane	O ₂ electrode	10	{ Schubert et al. (1985a)
Lactate/pyruvate	cytochrome b ₂ + lactate dehydrogenase	membrane	Pt electrode (+0.25 V)	10	
NADH/NAD ⁺	HRP + glucose dehydrogenase	membrane	O ₂ electrode	60	
	lactate dehydrogenase	membrane + LMO	O ₂ electrode	220	Schubert et al. (1990)
Glutamate	glutamate dehydrogenase + alanine aminotransferase	membrane	modified carbon electrode	15	{ Schubert et al. 1986b
			O ₂ electrode	60	
	glutamate dehydrogenase + glutamate oxidase	membrane	O ₂ electrode	220	Wollenberger et al. (1989)

TABLE 13 (Continued)

Analyte	Enzymes	Form of application	Transducer	Amplification factor	References
ADP/ATP	pyruvate kinase + hexokinase	membrane with LDH+LMO	O ₂ electrode	220	Wollenberger (1987b)
Ethanol	alcohol oxidase + alcohol dehydrogenase	membrane	O ₂ electrode		Hopkins (1985)
Benzoquinone/ hydroquinone	cytochrome b ₂ + laccase	membrane	O ₂ electrode	500	Scheller et al. (1987b)
ATP	pyruvate kinase + hexokinase + lactate dehydrogenase + lactate oxidase	2 reactors	thermistor	1700	Kirstein et al. (1987)
Malate/ oxaloacetate	lactate monooxygenase + malate dehydrogenase	membrane	O ₂ electrode	3	Schubert et al. (1991)

Scheller et al. (1985a) applied the recycling principle in an FIA-integrated enzyme thermistor for lactate determination. The analyte was recycled between lactate oxidase (LOD) and lactate dehydrogenase (LDH) coimmobilized on porous glass. For the amplification of the measuring signal it was advantageous that both enzyme reactions are exothermic (LDH: $\Delta H = -47$ kJ/mol, LOD: $\Delta H = -100$ kJ/mol). The overall reaction is the oxidation of the cosubstrate of LDH, NADH, by oxygen whereas lactate and pyruvate act as catalysts. This 'NADH oxidase' reaction has a reaction enthalpy as large as -225 kJ/mol. A thousandfold amplification as compared with the simple LOD reaction was obtained. The detection limit was lowered from $1 \mu\text{mol/l}$ to 1 nmol/l . A similar enzyme reactor has been combined with a Clark-type oxygen electrode in an FIA manifold (Asouzu et al., 1987). The detection limit was 10 nmol/l lactate.

The LOD-LDH thermistor has been combined with a reactor containing coimmobilized hexokinase (HK) and pyruvate kinase (PK) (Kirstein et al., 1987, 1989). These enzymes carry out the recycling of ATP/ADP whereby pyruvate is formed, which is then recycled in the LOD-LDH system. Although the enthalpy of the PK reaction is positive, the sufficiently negative enthalpies of the other three enzyme reactions provide an amplification factor of 1700 and thus a detection limit for ADP of 10 nmol/l . A PK-HK thermistor alone provided a 30-fold amplification and a detection limit of $2 \mu\text{mol/l}$.

In enzyme electrodes, which are deliberately operated under conditions of diffusion control, the diffusion limits the sensitivity. Here, the coupling of cyclic enzyme reactions gives rise to a sensitivity enhancement by overcoming the limit set by diffusion. The excess of enzyme present in the membrane is included in the substrate conversion. On the other hand, the upper limit of linearity and the operational stability are decreased.

An enzyme electrode based on coimmobilized cytochrome b_2 and laccase (Scheller et al., 1987b) allows an explanation of the principle of substrate recycling in enzyme electrodes in greater detail (Fig. 100). The advantage of this system is that the cosubstrate, oxygen, as well as the analytes, hydroquinone and benzoquinone, are electrochemically active. This permits one to study different parts of the recycling process. Recycling of the analyte in the presence of the substrate of cytochrome b_2 , lactate, results in an increase in the sensitivity by a factor of 500 as compared with lactate-free operation. Under conditions that are optimal for laccase the analyte is almost completely in the oxidized state, i.e. it

is present in the form of benzoquinone. If the reaction of cytochrome b_2 is favored, increasing lactate concentration leads to an increasing regeneration of hydroquinone from the benzoquinone previously formed in the laccase-catalyzed reaction, i.e., the equilibrium is shifted to the hydroquinone side. Thus, by addition of known quantities of lactate a threshold value of the electrochemical hydroquinone signal can be established (Fig. 101). This adjustable breakthrough provides the function of a threshold switch.

The application of the membrane-immobilized LOD-LDH system in enzyme electrodes has been shown to give an amplification factor of 250 in the steady state measuring regime (Mizutani et al., 1985) and 4100 in the rate measurement (Wollenberger et al., 1987a). The latter sensor was capable of detecting as little as 1 nmol/l lactate. With this sensor the theoretically derived dependence of the amplification on enzyme loading has been confirmed (Table 14). With increasing loading with both enzymes the amplification factor, G , increases and the detection limit decreases. From the equation for the amplification factor (Kulys et al., 1986a):

$$G = k_1 \cdot k_2 \cdot d^2 / 2(k_1 + k_2) \cdot D$$

where $k_i = v_{\max}/K_M$, and with $K_M(\text{lactate, LOD}) = 7 \cdot 10^{-7} \text{ mol/cm}^3$, $K_M(\text{py-}$

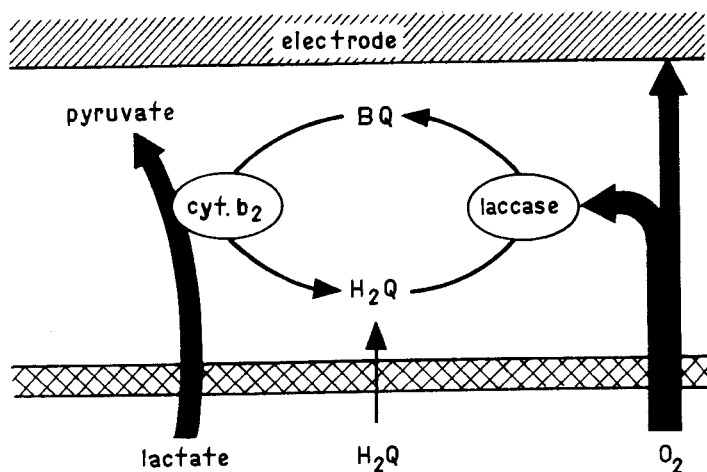


Fig. 100. Principle of an enzyme electrode with recycling of hydroquinone (H₂Q)/benzoquinone (BQ).

ruvate, LDH) = $1.4 \cdot 10^{-7}$ mol/cm³, and $d = 3 \cdot 10^{-3}$ cm, the characteristic diffusion time, τ , can be calculated:

$$\tau = d^2/D.$$

Theoretically, τ is independent of the enzyme loading. As shown in Table 14, the calculated values of τ have a maximum scattering factor of 5. If one takes account of uncontrollable changes in the membrane thickness resulting from sensor preparation, this is quite a good agreement.

In a recycling system using glutamate dehydrogenase and alanine aminotransferase the direct oxidation of the liberated NADH at an electrode modified with Meldola's Blue as well as the NADH oxidation by NMP⁺ and the subsequent detection of the oxygen consumption by

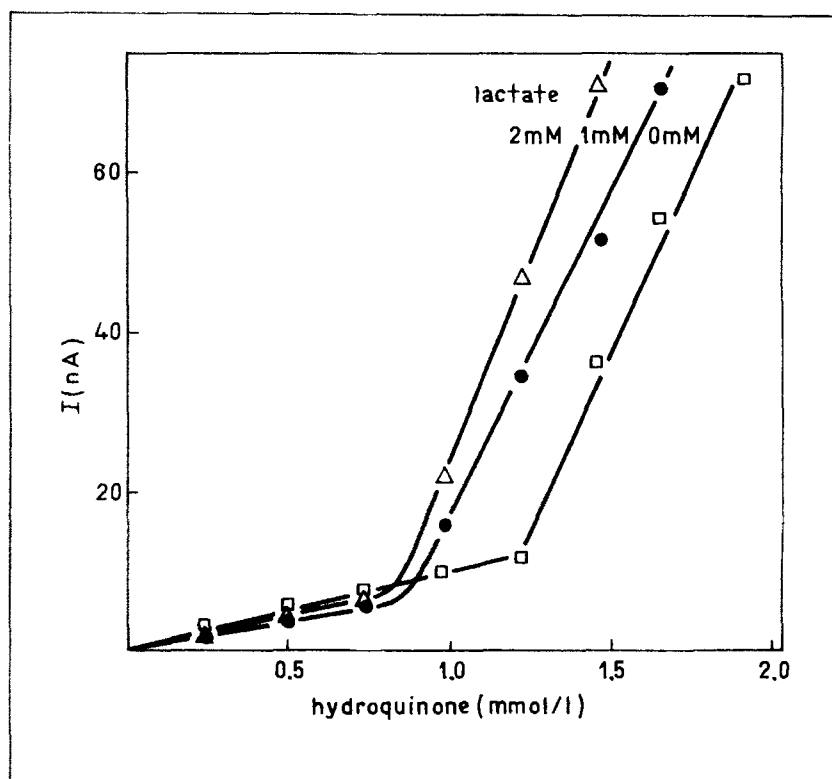


Fig. 101. Dependence of the hydroquinone signal of a cytochrome b₂-laccase electrode on the concentration of lactate in the solution.

TABLE 14

Effect of Enzyme Loading on Signal Amplification in the LOD-LDH Sensor

LOD, LDH (U/cm ²)	Detection limit (nmol/l)	Amplification factor	τ (s)
0.01	2000	2	60
0.1	100	50	150
1.0	100	100	30
10.0	1	4100	123

NMPH reoxidation have been used as the measuring signal (Schubert et al., 1986b). In the latter system the amplification was significantly higher since the NAD^+ regeneration proceeds in the whole volume of the enzyme membrane whereas the electrochemical oxidation of NADH takes place only at the electrode surface.

Using a sensor with GOD and GDH for glucose measurement, Schubert et al. (1985a) obtained an amplification factor of 10. This enzyme system is identical to the one employed in a competition sensor for NAD^+ determination (Pfeiffer et al., 1980) as described in Section 3.2.2, with the exception that the operating conditions are chosen so that the backward reaction of GDH is favored. In the competition mode the sensitivity to glucose is diminished and the upper limit of the linear range increased; in the recycling mode the opposite situation is obtained. In this way the qualitative characteristics of sensors based on coupled enzyme reactions can be manipulated by an appropriate adjustment of the measuring conditions.

The determination of both substrates of a recycling system, i.e., of S and P in Fig. 99, has been attempted by using cytochrome b_2 and LDH (Schubert et al., 1985a). At a tenfold amplification for lactate the sensitivities for lactate and pyruvate are almost identical. By investigating a recycling system for ATP and ADP using HK and PK (Fig. 102), Wollenberger et al. (1987b) have shown that the sensitivities towards both substrates of a cycle are not necessarily the same. For ADP an amplification factor of 220 and a detection limit of 0.25 $\mu\text{mol/l}$ were found; in contrast, ATP concentrations as low as 0.1 $\mu\text{mol/l}$ could still be assayed. In this sensor a recycling system has been coupled with an enzyme sequence (lactate monooxygenase and LDH) by coimmobilizing all the necessary enzymes in one membrane. It was thus demonstrated that enzymatic amplification sensors do not necessarily require recy-

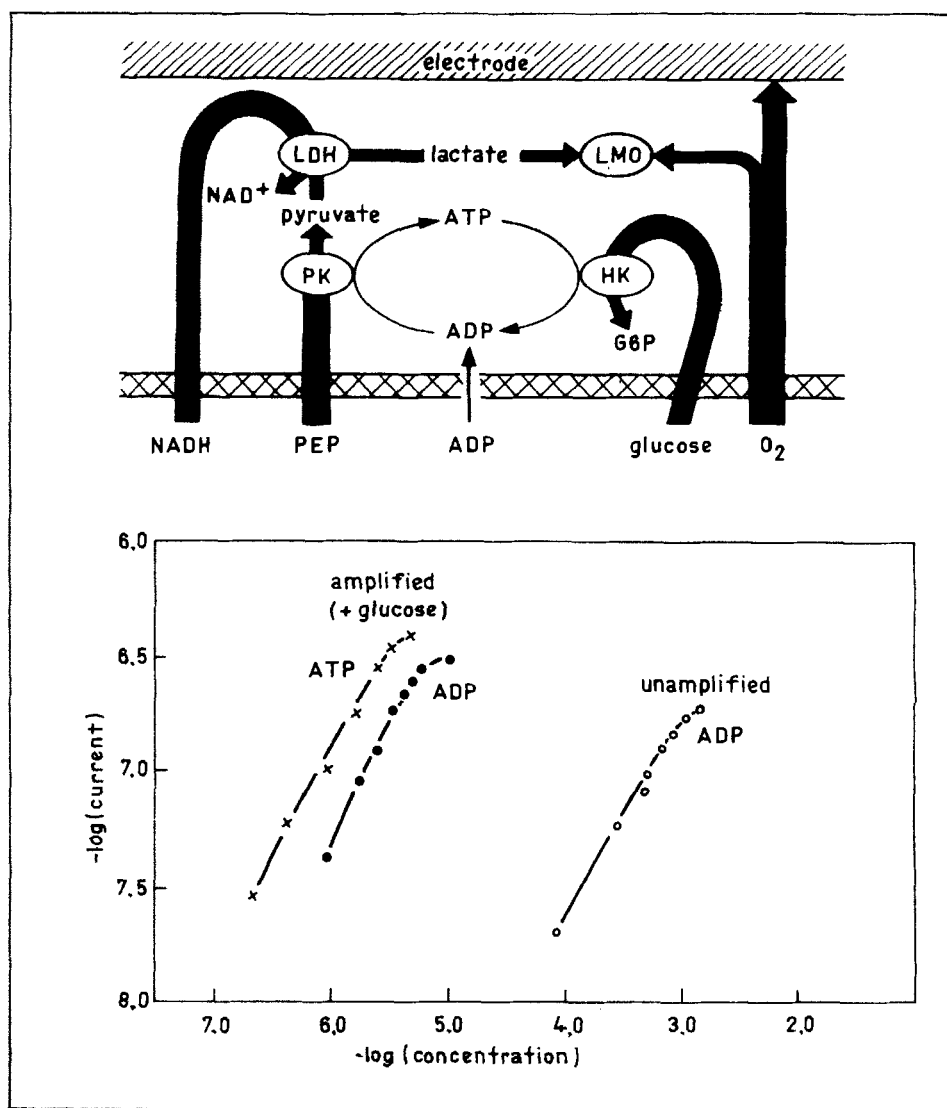


Fig. 102. Schematic representation and measuring curves of a recycling sensor for ADP and ATP with pyruvate measurement via the sequence lactate dehydrogenase-lactate monooxygenase. PEP = phosphoenolpyruvate. (Redrawn from Wollenberger et al., 1987a).

clinging systems producing electrode-active species. In the future the combination of several cycles, e.g., the kinase cycle with the LOD-LDH cycle as illustrated in Fig. 103, might lead to enzyme electrodes capable of detecting analytes below the nanomolar concentration range.

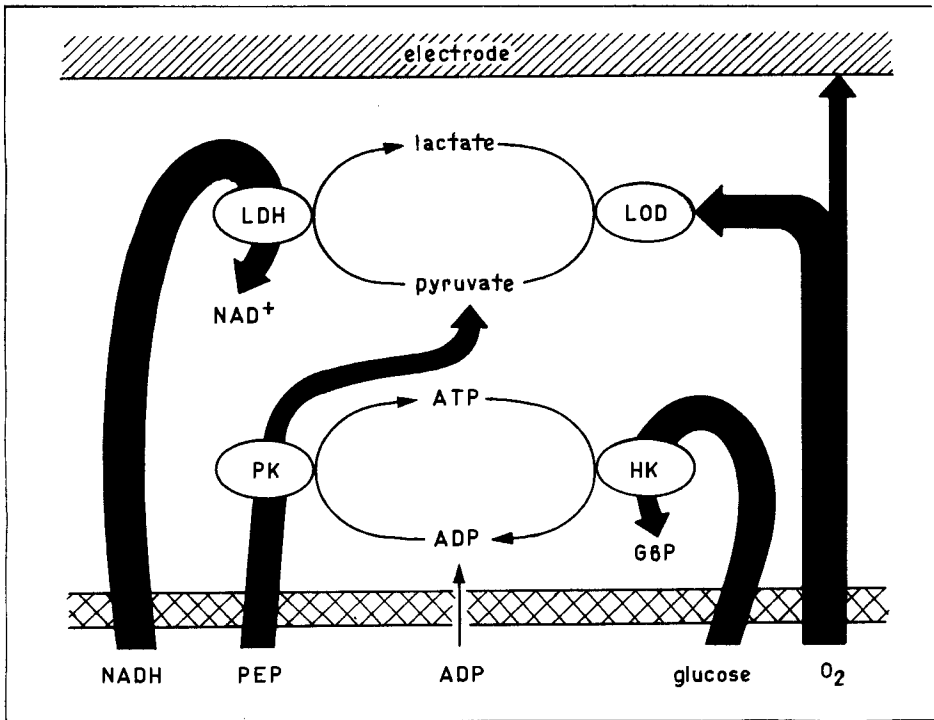
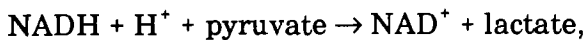
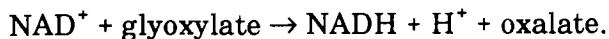


Fig. 103. Scheme of a double recycling sensor for ADP and ATP. (Redrawn from Wollenberger et al., 1987a).

The principle of enzymatic amplification can be drastically simplified by conducting the two partial reactions of the cycle with *only one* enzyme. Using this approach, Schubert et al. (1990) developed an LDH sensor for NADH determination. The enzyme was immobilized in a gelatin membrane and coupled to an oxygen probe, where it catalyzes the oxidation of NADH by pyruvate:



and, in the presence of glyoxylate, the reduction of the NAD⁺ formed:



During this coupled reaction in the membrane large amounts of lactate and oxalate are formed which can be indicated by coupled lactate monooxygenase or oxalate oxidase. Using lactate monooxygenase an amplification factor for NADH of 220 was obtained.

A less effective alternative to enzymatic recycling is recycling between the enzyme reaction and an appropriate electrode reaction, as has been described by Wasa et al. (1984b). Laccase was fixed by glutaraldehyde to a carbon electrode impregnated with epoxy resin. The substrates p-phenylene diamine and noradrenaline were oxidized by laccase and the reaction products indicated amperometrically by reduction at a potential of -0.1 V vs SCE. The substrate recycling gave rise to an amplification of the signal by a factor of between 10 and 20. This principle has been superseded by sensors using electrochemical (Mosbach et al., 1984) and chemical (Kulys and Malinauskas, 1979b) cofactor regeneration. The latter authors designed a recycling system for NAD^+ and NADH using alcohol dehydrogenase and NMP^+ . A similar approach has been employed by Lutter (1988) for the assay of alkaline phosphatase at an LDH electrode. NADP^+ was dephosphorylated by alkaline phosphatase, and the NAD^+ formed was recycled between membrane-immobilized LDH and NMP^+ . The oxygen consumption was measured amperometrically.

3.3 BIOSENSORS USING HIGHER INTEGRATED BIOCATALYSTS

The application by Divies (1975) of intact microbial cells in place of isolated, purified enzymes in a biosensor for ethanol determination initiated an intense search for novel biocatalytic materials useful for analysis. At the time of writing about 250 publications have appeared concerning the utilization of biocatalytically active systems on a higher level of integration (higher integrated systems, HIS). As might be expected, most of them deal with microorganisms, since they are the main sources of enzymes. However, cell organelles and tissue slices have also been used. Recent investigations directed at the employment of nerve cells and complete organisms demonstrate that this field continues to be an expanding area of research.

In addition to the comparatively simple and cheap preparation of the biocatalyst, HIS sensors are characterized by a number of advantages which challenge the traditional enzyme electrode concept. Owing to the native, 'evolution-optimized' environment, enzymes in HIS are usually more stable than isolated ones. The presence of physiologically interacting enzymes enables the construction of biosensors conducting multistep reactions under optimal conditions. The activity of microbial sensors can be regenerated by feeding with nutrients. Furthermore, it is possible to induce desirable enzyme activities and transport systems in the cell.

On the other hand, owing to the high diffusion resistance of cell and subcellular membranes, sensors based on HIS compare less favorably with enzyme sensors with respect to sensitivity and measuring time. Figure 104 presents a comparison of the basic characteristics of HIS sensors with those of enzyme sensors.

Since a large number of metabolic processes take place in HIS, HIS sensors generally appear less selective than enzyme electrodes. On the other hand, this property permits the detection of group effects (so-called complex variables), the measurement of whole classes of substances via their action on certain cell loci or on the metabolism as a whole, and the characterization of the biocatalytic systems themselves. The potentials of HIS sensors have been further expanded by the hybrid sensor technique, in which the substance to be determined is coupled to a given metabolic pathway by additional, coimmobilized enzymes.

The following sections are devoted to HIS sensors which will be presented according to the increasing level of integration and illustrated by selected examples. Since hybrid sensors have been constructed with

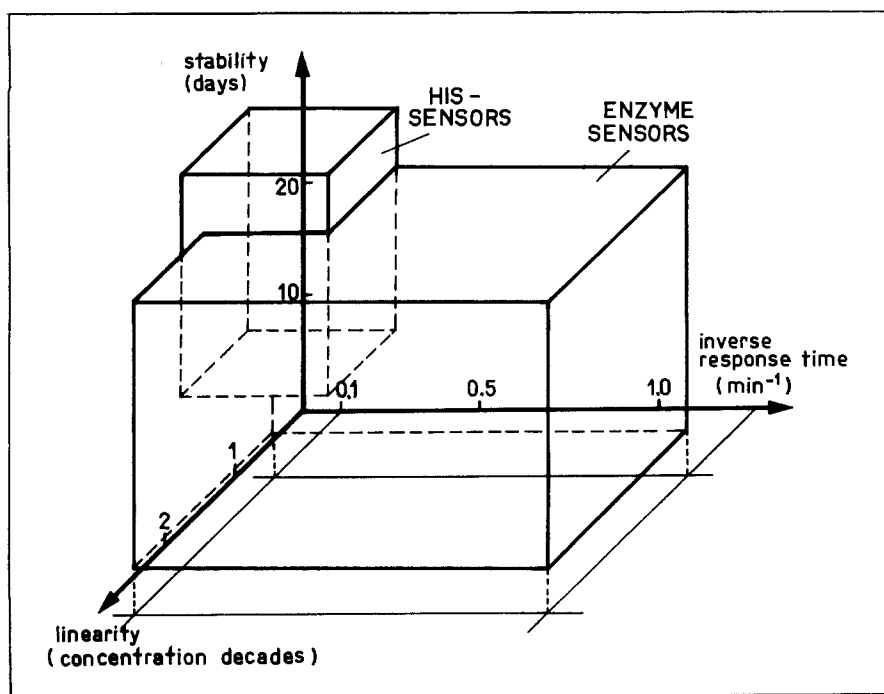
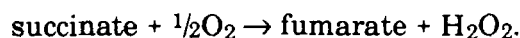
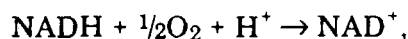


Fig. 104. Comparison of the characteristic parameters of enzyme sensors and sensors using higher integrated biocatalytic systems (HIS).

nearly all kinds of biocatalytic material, no special attention will be paid to this sensor type.

3.3.1 Cell Organelles

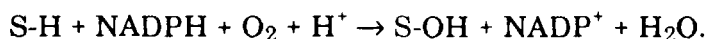
The employment of cell organelles has been limited to mitochondrial and microsomal fractions (Table 15). As early as 1976, Guilbault et al. coupled pig heart mitochondrial electron transfer particles (ETP) with an oxygen electrode. This development has been further pursued by Aizawa et al. (1980c). Decoupling of the electron transfer from oxidative phosphorylation makes ETP catalyze the following reactions:



Having a lifetime of two weeks, the sensor was capable of determining NADH and succinate with a linear range up to 0.13 and 0.15 mmol/l, respectively. Competitive inhibition of the mitochondrial succinate dehydrogenase activity by malonate resulted in a totally selective sensor for NADH. In this way the selectivity enhancement of HIS sensors by inhibition of interfering metabolic routes was demonstrated.

The high glutaminase (EC 3.5.1.2) activity of porcine kidney mitochondria has been utilized in a glutamine sensor based on an ammonia gas-sensing electrode (Arnold and Rechnitz, 1980a). The sensor has been compared with sensors using isolated glutaminase, porcine kidney slices, and *Sarcina flava* bacteria. No significant differences were found with respect to sensitivity, measuring range, and response time. The lifetimes of the enzyme and organelle sensors, 1 day and 10 days, were considerably lower than those of the bacteria and tissue slice sensors of 20 and 30 days. The mitochondrial sensor required an elaborate operating medium to maintain the mechanical integrity of the biocatalyst.

The microsomal fraction of liver contains a monooxygenase system consisting of cytochrome P-450 (EC 1.14.14.1), NADPH-cytochrome P-450 reductase (EC 1.6.2.4), and phospholipid. This system catalyzes the hydroxylation of a large number of both foreign and endogenous compounds in a mixed-function oxidation reaction using molecular oxygen and NADPH or NADH as electron donors:

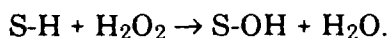


These can be replaced by hydrogen peroxide:

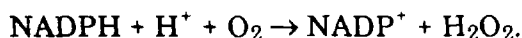
TABLE 15

Organelle Sensors

Biocatalyst	Substrate	Species detected	Linear range (mmol/l)	Response time (min)	Lifetime (d)	References
Mitochondrial ETP	succinate	O ₂	1.1...10.0	1	7	Guilbault (1976)
	NADH	O ₂	0.01...0.13	6	14	Aizawa et al. (1980c)
	succinate	O ₂	0.01...0.15	6	14	
Mitochondria	glutamine	NH ₃	0.11...5.5	7	10	Arnold and Rechnitz (1980a)
Microsomes	aniline	p-aminophenol	0.05...0.5	4	3	Schubert et al. (1980)
	NADPH	O ₂	0.05...1.0	3	14	Schubert et al (1982a)
	NADH	O ₂		3	14	Schubert et al (1982a)
	glucose-6-phosphate	O ₂		3	14	Schubert et al (1982a)
	sulphite	O ₂	0.06...0.34	10	2	Karube et al. (1983)
Microsomes + glucose-6-phosphate dehydrogenase + hexokinase	ATP	O ₂	0.01...0.15	3	14	Schubert et al (1982b)
Microsomes + isocitrate dehydrogenase	D-isocitrate	O ₂	0.02...0.3	3	7	Schubert et al (1982b)
Microsomes + GOD	aniline	p-aminophenol	0.05...0.5	4	3	Schubert et al (1982b)
Microsomes + lactate dehydrogenase	L-lactate	O ₂	1.0...10.0	6		Schubert (1983)



The system is also responsible for aerobic oxidation of NADPH in the absence of substrates that can be hydroxylated:



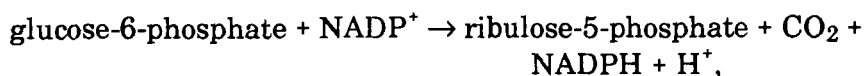
The hydroxylase as well as the NADPH oxidase have been studied with regard to their applicability in biosensors. For this purpose, intact liver microsomes have been immobilized in gelatin membranes with relatively high activity yield (Schubert et al., 1980). Aniline has been chosen as the model substrate for the design of a microsomal hydroxylase electrode because the reaction product, p-aminophenol, can be electrochemically oxidized to the respective iminoquinone. At the required overvoltage of +0.25 V neither NADPH nor H_2O_2 , one of which has to be present as cofactor, is cooxidized at the electrode. Both the NADPH-dependent and the hydrogen peroxide-dependent hydroxylation have been utilized in aniline sensors (Fig. 105). For the latter, GOD was coimmobilized with microsomes and glucose was added to the measuring solution. In this hybrid sensor the nascent hydrogen peroxide liberated in the GOD-catalyzed reaction is consumed by cytochrome P-450, thus minimizing the inactivation of the hemoprotein by peroxide. The anodic oxidation currents of both the microsomal and the hybrid sensor were proportional to aniline concentration up to 0.5 mmol/l.

The electrochemical indication of cytochrome P-450-catalyzed hydroxylation should also be possible by oxygen or H_2O_2 sensors. However, because of the high NADPH oxidase activity of microsomes the amount of oxygen consumed does not correlate directly with the substrate concentration. Furthermore, the addition of the usually hydrophobic substrates may change the solubility of oxygen in the measuring solution. On the other hand, hydrogen peroxide indication is not advisable because many cytochrome P-450 substrates are electrochemically oxidized at the H_2O_2 oxidation potential. Therefore the principle of electrochemical product indication described above seems to be a preferable basis for cytochrome P-450 hydroxylase sensors, though being limited to substrates which are hydroxylated to electrochemically distinct products, such as aniline, codeine (Weber and Purdy, 1979), paracetamol (Miner et al., 1981), and some primary arylamines (Sternson, 1974).

Some of the above-mentioned characteristics of HIS biosensors can be demonstrated by considering the application of the liver microsomal NADPH oxidase activity in such sensors. The coupling of gelatin-immobilized

bilized microsomes with a Clark-type oxygen probe resulted in a sensor for NADPH and NADH (Schubert et al., 1982a). The sensor had a functional stability of 14 days which, in comparison with the stability of isolated cytochrome P-450 of only a few hours, indicates the stabilizing effect of the biological environment.

Since, in the microsomal membrane, two NADP⁺-dependent dehydrogenases are present that catalyze the sequential oxidation of glucose-6-phosphate (G6P) to ribulose-5-phosphate:



the sensor was also useful for G6P measurement. According to the reaction scheme (Fig. 106), two molecules of NADP⁺ are reduced per molecule of G6P. The sensor was thus almost twice as sensitive to G6P as it was to NADPH. The lifetime of the endogenous four-enzyme sequence used (hexose-6-phosphate dehydrogenase, 6-phosphogluco-

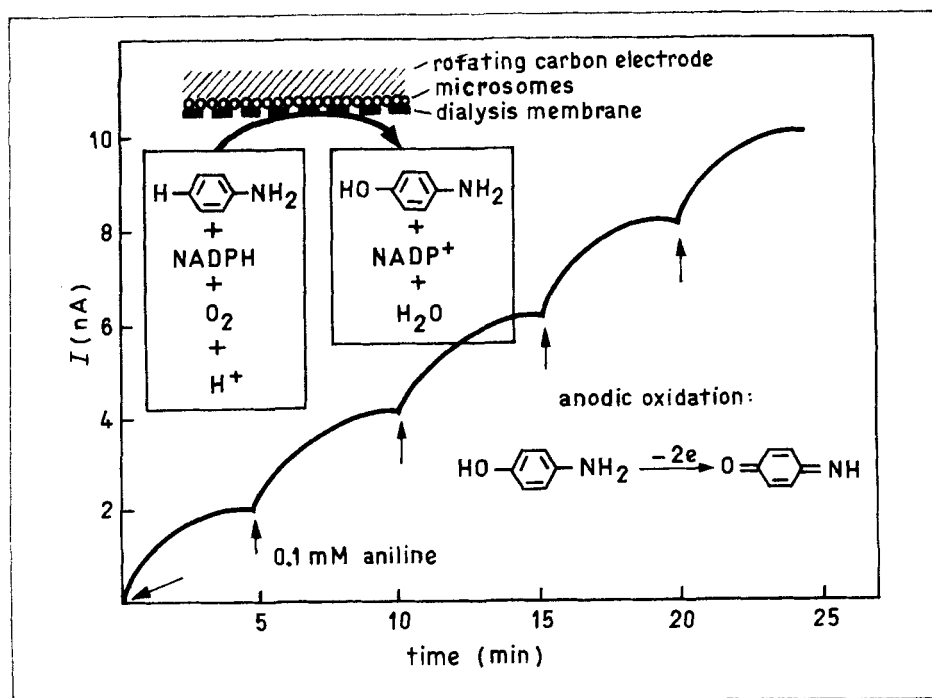


Fig. 105. Construction and response curve of an organelle electrode for aniline based on immobilized liver microsomes. (Redrawn from Mohr et al., 1984).

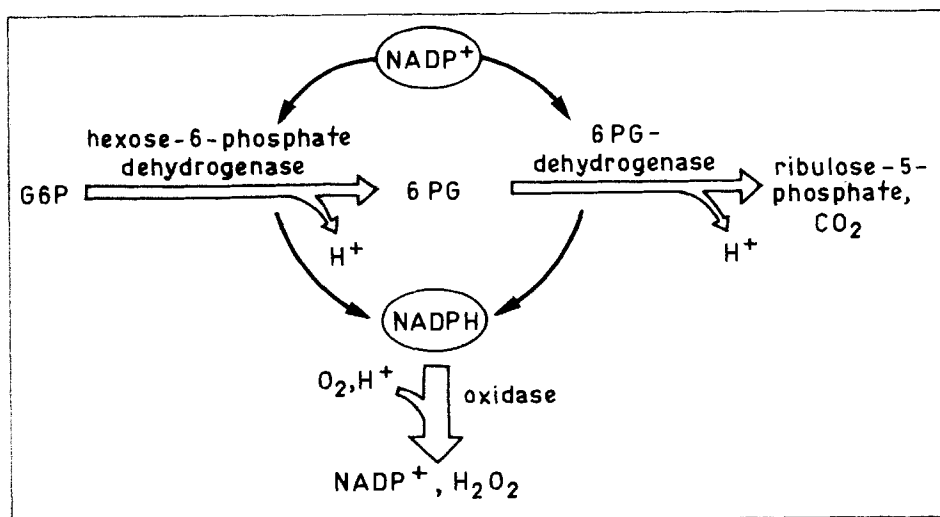


Fig. 106. Coupled conversion of glucose-6-phosphate in liver microsomes. 6PG = 6-phosphogluconate. (Redrawn from Schubert et al., 1982a).

nate dehydrogenase, NADPH-cytochrome P-450 reductase, cytochrome P-450) was 14 days.

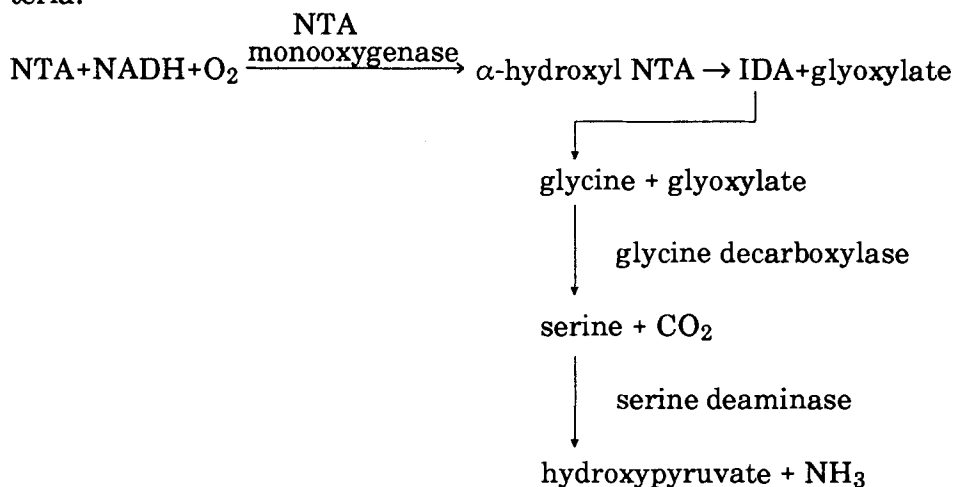
The widespread use of dehydrogenase reactions in clinical chemistry prompted further investigation of the coupling of other enzymes with the microsomal NADPH oxidase in hybrid electrodes (Schubert et al., 1982b). Thus, sensors for ATP and isocitrate have been assembled (Table 15), which indicates the usefulness of this concept.

3.3.2 Microorganisms

Microbial sensors differ from other HIS sensors in that they contain living matter. This makes them regenerable, but can also give rise to undesired alterations during sensor operation. Table 16 gives an overview of microbial sensors for substrate determination. In most cases only one activity out of the large arsenal of enzymes has been utilized, frequently a deaminase or decarboxylase. Microbial dehydrogenases have been coupled with electrochemical processes via mediators (Kulys and Kadziauskiene, 1978; Wollenberger et al., 1980b) and lipoic acid (Planchard et al., 1988).

Kobos and Pyon (1981) were the first to use a complex multistep reaction in a microbial electrode. The growing of pseudomonades in a medium containing nitrilotriacetic acid (NTA) as the sole source of

carbon and nitrogen induced the following metabolic route in the bacteria:



IDA = iminodiacetate

The cells were immobilized on an ammonia gas-sensing electrode. The potential of the resulting microbial sensor was linearly dependent on the concentration of NTA between 0.1 and 1 mmol/l. Intermediates of NTA metabolism, such as glycine and serine, were measured with the same sensitivity.

Another complex reaction sequence has been employed in a sulfate sensor composed of *Desulfovibrio desulfuricans* cells and a sulfide selective electrode (Kobos, 1986). The sensor responded linearly to sulfate between 0.04 and 0.7 mmol/l and was stable for 10 days. Since oxygen irreversibly inhibits the enzymes used, the sensor had to be operated under strictly anaerobic conditions.

Burstein et al. (1986) have shown that in addition to induction and inhibition mechanical and chemical changes of the cell structure effect the selectivity of microbial sensors. *Escherichia coli* cells were treated by ultrasound, extrusion, or crosslinked with glutaraldehyde before used in sensors for D- and L-lactate, succinate, L-malate, 3-glycerophosphate, pyruvate, NADH, and NADPH. The treatment caused different selectivity of the respiratory chain and thus permitted an increase in the specificity of the respective sensors.

Microbial hybrid electrodes using nitrifying bacteria fixed together with NH_3 -producing enzymes on an oxygen probe have been developed for the determination of urea (Okada et al., 1982) and creatinine (Kubo et al., 1983a). For urea assay a membrane containing immobilized

TABLE 16

Microbial Sensors for Substrate Determinations

Substrate	Microorganism	Detection mode	Measuring range (mmol/l) ¹	References
D-Glucose	<i>Pseudomonas fluorescens</i>	O ₂ (amp.)	0.0125–0.125	Karube et al. (1979b)
	<i>Bacillus subtilis</i>	O ₂ (amp.)	0–0.6	Riedel et al. (1984)
	<i>Saccharomyces cerevisiae</i>	O ₂ (amp.)	0.05–0.7	Mascini and Memoli (1986)
		CO ₂ (pot.)	1.0–1.5	
	<i>Escherichia coli</i>	lipoic acid	0.02–0.4	Planchard et al. (1988)
	mixed population	pH (ISFET)	0–3.9	Hanazato and Shiono (1983)
D-Glucose	} <i>Brevibacterium lactofermentum</i>	O ₂ (amp.)	0–1.0	Hikuma et al. (1980a)
D-Fructose			0–1.0	
Sucrose			0.8	
Maltose	<i>B. subtilis</i>	O ₂ (amp.)	0–0.5	Riedel et al. (1984)
Hexoses	plaque cells	pH (pot.)		Grobler and Rechnitz (1980)
Formate	<i>Clostridium butyricum</i>	H ₂ (fuel cell)	0–22	Matsunaga et al. (1980b)
	<i>Pseudomonas oxalaticus</i>	CO ₂ (pot.)	0.1–2.0	Ho and Rechnitz (1985)
Acetate	<i>Trichosporon brassicae</i>	O ₂ (amp.)	0–0.9	Hikuma et al. (1979b)
Pyruvate	<i>Streptococcus faecium</i>	CO ₂ (pot.)	0.02–32	Di Paolantonio and Rechnitz (1983)
L-Lactate	<i>Hansenula anomala</i>	[Fe(CN) ₆] ⁴⁻	0–30	Vincké et al. (1985a)
		(amp.)	0.01–0.5	Hauptmann (1985)
			0–8.0	Kulys and Kadziuskiene (1978)
			0.02–0.5	Racek and Musil (1987)
		(pot.)	0.4–2.0	Vincké et al. (1985a)
	<i>E. coli</i>	O ₂ (amp.)	0–3.0	Burstein et al. (1986)

TABLE 16 (Continued)

Substrate	Microorganism	Detection mode	Measuring range (mmol/l) ¹	References
D-Lactate			0–10	
Succinate			0–10	Burstein et al.
L-Malate	<i>E. coli</i>	O ₂ (amp.)	0–20	(1986)
3-Glycero-phosphate			0–5.0	
L-Glutamate	<i>E. coli</i>	CO ₂ (pot.)	0.6–5.5	Hikuma et al. (1980c)
	<i>B. subtilis</i>	O ₂ (amp.)	0–0.15	Riedel and Scheller (1987)
L-Glutamine	<i>Sarcina flava</i>	NH ₃ (pot.)	0.1–1.0	Rechnitz et al. (1978)
L-Aspartate	<i>B. cadaveris</i>	NH ₃ (pot.)	0.3–7.0	Kobos and Rechnitz (1977)
L-Asparagine	<i>Serratia marescens</i>	NH ₃ (pot.)	1.0–9.3	Vincké et al. (1983a)
L-Arginine	<i>Streptococcus faecium</i>	NH ₃ (pot.)	0.05–1.0	Rechnitz et al. (1977)
L-Lysine	<i>E. coli</i>	CO ₂ (pot.)	0.07–0.7	Suzuki and Karube (1980)
L-Serine	<i>Clostridium acidurici</i>	NH ₃ (pot.)	0.18–16	Di Paolantonio et al. (1981)
L-Tryptophan	<i>Pseudomonas fluorescens</i>	O ₂ (amp.)	0.0004–0.07	Vincké et al. (1985b)
L-Cysteine	<i>Proteus morganii</i>	H ₂ S (pot.)	0.05–0.9	Jensen and Rechnitz (1978)
L-Histidine	<i>Pseudomonas</i> sp.	NH ₃ (pot.)	0.1–3.0	Walters et al. (1980)
L-Tyrosine	<i>Aeromonas phenologenes</i>	NH ₃ (pot.)	0.08–1.0	Di Paolantonio and Rechnitz (1982)
Methanol	bacteria	O ₂ (amp.)	0.06–0.7	Karube et al. (1980a)
Ethanol	<i>T. brassicae</i>	O ₂ (amp.)	0.05–0.5	Hikuma et al. (1979a)
	<i>S. cerevisiae</i>	[Fe(CN) ₆] ⁴⁻ (pot.)	0.1–10	Pascual et al. (1982)
	<i>Acetobacter xylinum</i>	O ₂ (amp.)	0–0.4	Divies (1975)

TABLE 16 (Continued)

Substrate	Microorganism	Detection mode	Measuring range (mmol/l) ¹	References
	<i>Acetobacter aceti</i>	pH (ISFET)	3–70	Kitagawa et al. (1987)
Methane	<i>Methylomonas flagellata</i>	O ₂ (amp.)	0.005–6.6	Karube et al. (1982a)
O ₂	<i>Photobacterium fischeri</i>	light intensity	0.00004–0.01	Lloyd et al. (1981)
CO ₂	thermophilic bacteria	O ₂ (amp.)	3–12%	Suzuki et al. (1988b)
Phenol	<i>T. cutaneum</i>	O ₂ (amp.)	0–0.15	Neujahr and Kjellén (1979)
Thiamine	<i>S. cerevisiae</i>	O ₂ (amp.)	0.01–0.5 µg/ml	Mattiasson et al. (1982)
Nicotinic acid	<i>Lactobacillus arabinosa</i>	pH (pot.)	0.4–40	Matsunaga et al. (1978)
Nicotinamide	<i>B. pumilus</i>	NH ₃ (pot.)	0.28–14.	Vincké et al. (1984)
	<i>E. coli</i>	NH ₃ (pot.)	0.18–20	
NAD ⁺	<i>E. coli</i> (+ NADase)	NH ₃ (pot.)	0.25–2.5	Riechel and Rechnitz (1978)
L-Ascorbate	<i>Enterobacter agglomerans</i>	O ₂ (amp.)	0.004–0.7	Vincké et al. (1985c)
Cholesterol	<i>Nocardia erythropolis</i>	O ₂ (amp.)	0.015–0.13	Wollenberger et al. (1980a)
Androstenedione; testosterone	<i>Nocardia opaca</i>	DCPIP (amp.)	0.0015–0.1	Wollenberger et al. (1980b)
Cephalosporin	<i>Citrobacter freundii</i>	pH (pot.)	50–300 µg/ml	Matsumoto et al. (1979)
Nystatin	<i>S. cerevisiae</i>	O ₂ (amp.)	0–54 U/ml	Karube et al. (1979a)
Nitrilotriacetic acid	<i>Pseudomonas</i> sp.	NH ₃ (pot.)	0.1–0.7	Kobos and Pyon (1981)
Ammonia	<i>Nitrosomonas europaea</i>	O ₂ (amp.)	0–0.08	Hikuma et al. (1980b)
	activated sludge	O ₂ (amp.)	0.005–4.5	Karube et al. (1981a)
	<i>B. subtilis</i>	O ₂ (amp.)	0–0.15	Riedel et al. (1985a)
Nitrate	<i>Azotobacter vinelandii</i>	NH ₃ (pot.)	0.01–0.8	Kobos et al. (1979)

TABLE 16 (Continued)

Substrate	Microorganism	Detection mode	Measuring range (mmol/l) ¹	References
Nitrite	<i>Nitrobacter</i> sp.	O ₂ (amp.)	0.01–0.54	Karube et al. (1982d)
Herbicides	activated sludge	O ₂ (amp.)	0.01–5.0	Okada et al. (1983)
	<i>Synechococcus</i>	[Fe(CN) ₆] ⁴⁻ , p-benzoquinone (amp.)	20 ppb–2 ppm	Rawson et al. (1987)
Urea	activated sludge (+ urease)	O ₂ (amp.)	2.0–200	Okada et al. (1982)
			1.5–10	Kubo et al. (1983b)
	<i>Proteus mirabilis</i>	NH ₃ (pot.)	0.5–50	Vincké et al. (1983b)
Creatinine	<i>Nitrosomonas</i> sp. + <i>Nitrobacter</i> sp. (+ creatininase)	O ₂ (amp.)	0.4–76	Kubo et al. (1983a)
Uric acid	<i>Pichea membranaefaciens</i>	CO ₂ (pot.)	0.1–2.5	Kawashima et al. (1984)
	<i>Altenaria tennisi</i>	O ₂ (amp.)	0.025–0.5	Wollenberger (1981)
Sulphide	<i>Chromatium</i> sp.	H ₂ (amp.)	0.4–3.5	Matsunaga et al. (1984b)
Sulphate	<i>Desulfovibrio desulfuricans</i>	H ₂ S (pot.)	0.04–0.7	Kobos (1986)
Monomethylsulphate	<i>Hyphomicrobium</i>	pH (pot.)	25–630	Schär and Ghisalba (1985)
Phosphate	<i>Chlorella vulgaris</i>	O ₂ (amp.)	8.0–70	Matsunaga et al. (1984a)
Aspartame	<i>B. subtilis</i>	O ₂ (amp.)	0.07–0.6	Renneberg et al. (1985)
Angiotensin GnRH ²	<i>B. subtilis</i>	O ₂ (amp.)	0–15	Riedel et al. (1988)
	<i>B. subtilis</i>	O ₂ (amp.)	0–30	Riedel et al. (1988)
Immuno-globulin G	<i>Staphylococcus aureus</i>	O ₂ (amp.)	0.1–10 µg/ml	Aizawa et al. (1983)
α-Amylase	<i>B. subtilis</i> (+ glucoamylase)	O ₂ (amp.)	0–1.5 U/ml	Renneberg et al. (1984)

¹if not otherwise stated; ² gonadotrophine-releasing hormone

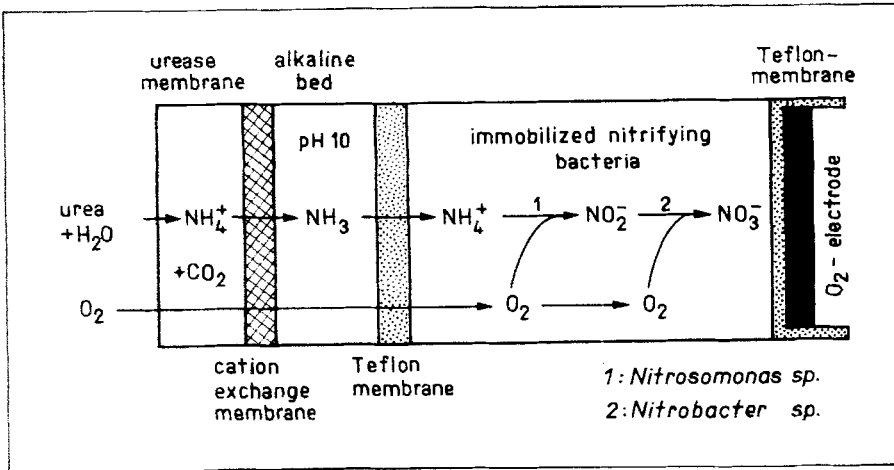


Fig. 107. Bacterial hybrid electrode for determination of urea. (Redrawn from Okada et al., 1982).

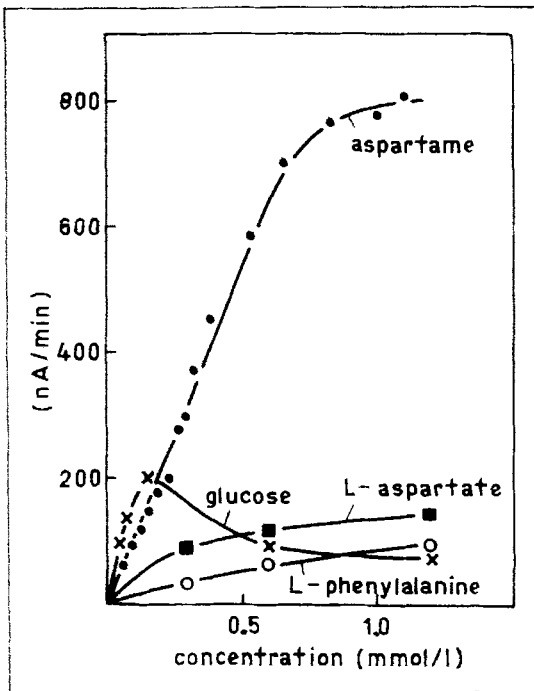


Fig. 108. Concentration dependence of the signals for aspartame, its amino acid constituents, and glucose of a sensor containing *B. subtilis* cells grown on aspartame. (Redrawn from Renneberg et al., 1985).

urease was separated by a Teflon membrane from the layer of immobilized bacteria (Fig. 107). This configuration ensured that apart from oxygen and ammonia gas no substrate was able to diffuse to and be metabolized in the bacterial layer. Owing to the high diffusion resistance of this multimembrane system the sensor was rather slow to respond and was insensitive, the lower detection limit for urea being 2 mmol/l. Nitrifying bacteria have also been used in sensors for ammonia (Hikuma et al., 1980b), nitrate (Karube et al., 1981a), and nitrite (Karube et al., 1982d). The selectivity of these sensors was also increased by using an internal gas-permeable membrane.

Riedel et al. (1985a) proposed utilization of the acceleration of glucose respiration by *Bacillus subtilis* resulting from active NH_4^+ permeation in a sensor for the determination of ammonium ion. The normally repressed NH_4^+ permease system of the cells was activated by nutrient limitation. The cells were fixed to the tip of an oxygen electrode. The sensor exhibited constant sensitivity for more than 12 days.

Another *B. subtilis* sensor has been devised by Renneberg et al. (1985). These authors induced permeation and assimilation systems for aspartame by growing the cells on this artificial sweetener. An oxygen electrode equipped with the cells was capable of assaying aspartame in the concentration range 0.07–0.6 mmol/l (Fig. 108). The sensitivity for the two aspartame amino acid constituents, aspartate and phenylalanine, amounted to 20% and 10% of the sensitivity for the dipeptide. The sensitivity for glucose was also low. Increasing the sensor specificity by induction has also been used for the design of sensors for other peptides and for hormones (Riedel et al., 1988).

A selectivity enhancement by inhibition of interfering reactions has been achieved by Riedel and Scheller (1987), who added chloromercuribenzoate and NaF to the measuring solution of a *B. subtilis* sensor for glutamate to block glucose permeation and assimilation. In this manner the interference of glucose in the glutamate measurement was completely eliminated.

Renneberg et al. (1984) described a microbial hybrid sensor for α -amylase assay. A membrane with coimmobilized *B. subtilis* cells and glucoamylase was attached to an O_2 electrode. Starch and the α -amylase sample were added to the measuring cell. Low-molecular weight products of the α -amylase-catalyzed starch hydrolysis diffuse into the biocatalytic membrane where they are cleaved by glucoamylase to glucose, which is assimilated by the bacteria. The sensor responded linearly to α -amylase up to 1.5 U/ml.

Grobler and Rechnitz (1980) reported the application of dental plaque in a biosensor for assay of the hexoses D-glucose, D-mannose, D-galactose, and D-fructose. The plaque cells were immobilized around the active tip of a pH electrode. The sensor did not respond to pentoses.

Rechnitz and his coworkers (Kobos and Rechnitz, 1977; Jensen and Rechnitz, 1978) demonstrated the regeneration of microbial sensors by using electrodes for L-aspartate and L-cysteine. The biocatalytic activity was restored by placing the spent electrodes back into the nutrient growth medium used for the culturing of the relevant bacterial strain. Fresh cells were grown *in situ* at the electrode surface so that the initial activity could be largely regenerated and the electrode lifetime extended. However, this method becomes self-limiting after a few regrowth cycles owing to the build-up of cellular debris on the electrode surface.

The coupling of microorganisms with pH sensitive FETs to produce sensors for glucose and alcohol has been studied by Hanazato and Shiono (1983) and Kitagawa et al. (1987). The transistor gate was covered by the cells entrapped in agar and alginate, respectively. The response times of these sensors were as long as 30 min. The alcohol sensor was separated from the measuring solution by a gas-permeable membrane in order to exclude disturbances by pH changes.

A microbial FET for the determination of alcohol has been constructed by Tamiya et al. (1988). The cell membrane of *Gluconobacter suboxydans*, which converts ethanol to acetic acid, was attached in calcium alginate to the gate of a pH-FET and covered by a nitrocellulose layer. The differential output versus a membrane-free reference gate was linearly related to the logarithm of the ethanol concentration up to 20 mg/l. The sensor responded to propanol and butanol with similar sensitivity, but not to methanol. The response time was 10 min. Below 30°C the sensor was stable for 40 h.

Microbial sensor systems for the detection of complex variables and for the characterization of microbes are listed in Table 17. Karube et al. (1981b, 1982c) developed microbial sensor-based methods for the screening of mutagens based on the monitoring of immobilized cell respiration with an O₂ electrode. In order to detect small effects on cell respiration only small amounts of cells were used. In one of the procedures (Karube et al., 1981b) two microbial electrodes using a recombinant deficient strain, *B. subtilis* (Rec⁻), and a wild strain of *B. subtilis* were employed. The principle of the sensor system was based on the inhibitory action of mutagens on the respiration of Rec⁻ cells in a glucose-containing solution. When mutagens were added to the solution, the current of the Rec⁻ electrode gradually

TABLE 17

Microbial Sensors for Complex Variables and for Characterization of Microorganisms

Assay	Microbes	References
Biological oxygen demand	<i>Clostridium butyricum</i> <i>H. anomala</i> <i>Trichosporon cutaneum</i> <i>B. subtilis</i> , <i>T. cutaneum</i>	Karube et al. (1977a,b) Kulys and Kadiauskiene (1980) Hikuma et al. (1979c)
Assimilation test	several	Hikuma et al. (1980d)
Assimilable sugars	<i>Brevibacterium lactofermentum</i>	Hikuma et al. (1980a)
Mutagens	<i>B. subtilis</i> <i>Salmonella typhimurium</i>	Karube et al. (1981b) Karube et al. (1982c)
Cell populations	several	Matsunaga et al. (1980a); Nishikawa et al. (1982); Matsunaga and Namba (1984a, b)
Cell number	several	Matsunaga et al. (1981)
Physiological state	<i>B. subtilis</i>	Riedel et al. (1985b)
Differentiation gram-negative/gram-positive	several	Matsunaga and Nakajima (1985)

increased whereas that of the wild strain sensor remained constant because the wild strain cells were able to repair the induced DNA damage. Linear relations were obtained between the rate of the current increase of the Rec⁻ electrode and the mutagen concentration. With this assay the preliminary screening of mutagens was possible within 1 h. The minimum detectable concentrations were 2 µg/ml for captan and 1.6 µg/ml for AF-2. For conventional mutagen tests at least 2 days are needed.

With a mutant of *Salmonella typhimurium* the detection limit for mutagens was further decreased by orders of magnitude (Karube et al., 1982c). The principle of this assay is shown in Fig. 109. The mutant, which requires histidine for growth, was incubated in histidine-free nutrient medium with a mutagen. Some of the revertants formed were able to grow on the medium in the absence of histidine. A defined volume of the incubate was transferred to a membrane filter and fixed to an oxygen electrode. In the presence of glucose a current decrease due to the respiration of the cells was observed. When the substance to be tested was not mutagenic, no

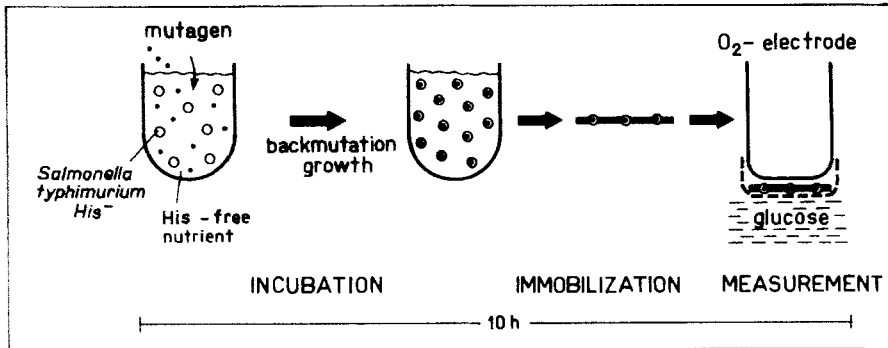


Fig. 109. Mutagenicity test using a microbial sensor.

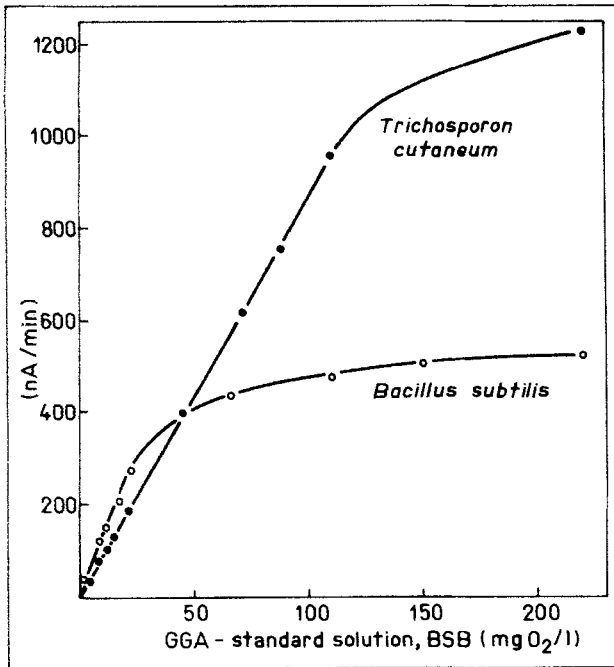


Fig. 110. Measuring curves of BOD sensors containing different microorganisms. Glucose-glutamic acid (GGA) standard solutions were used as BOD reference.

back-mutation occurred. Consequently, the cells were unable to grow and no respiration could be detected at the electrode.

The ability of microbes to degrade a multitude of organic compounds is utilized in sensors for the determination of the biological oxygen

demand (BOD). BOD is an important parameter for the evaluation of organically contaminated wastewater. The conventional BOD-test takes 5 days. Rapid BOD measurement has been shown to be feasible by applying immobilized cells coupled with oxygen electrodes. Such sensors have been assembled by using cells of *Clostridium butyricum* (Karube et al., 1977a,b), *Hansenula anomala* (Kulys and Kadziauskiene, 1980), *B. subtilis* (Riedel et al., 1987), and *Trichosporon cutaneum* (Suzuki and Karube, 1979; Riedel et al., 1987). The current-concentration dependence of a *B. subtilis* sensor for BOD is shown in Fig. 110. The *Trichosporon cutaneum* sensor is being used for wastewater control in industrial fermentation (see Section 5.4).

Karube et al. (1977c) proposed a biofuel cell containing *Clostridium butyricum* cells for BOD assay. The anodic oxidation current of hydrogen and formate anaerobically formed from organic compounds was used as the measuring signal.

Thermophilic bacteria have been employed in a BOD sensor for the monitoring of hot wastewater (Karube, 1988).

If the wastewater contains organic substances which are not degradable by a single microbial strain, activated sludges used for wastewater treatment can be taken for BOD sensor preparation (Strand and Carlson, 1984). Such mixed populations usually suffer from a poor stability.

Characterization of Microorganisms

For the electrochemical determination of cell populations Matsunaga et al. (1980a) made use of the phenomenon that bacteria can be anodically oxidized. In a *B. subtilis* cell suspension the current difference between a bare electrode and a membrane-covered electrode was measured; $0.2 \cdot 10^{-9}$ – $2 \cdot 10^{-9}$ cells could be detected with good reproducibility. In a similar manner, cell populations have been determined at electrodes modified with 4,4'-bipyridyl (Matsunaga and Namba, 1984a) or dyes (Nishikawa et al., 1982), and by cyclic voltammetry (Matsunaga and Namba, 1984b).

Sensors for the characterization of the microorganisms themselves, which are likewise based on their low selectivity, use the detection of the respirative activity of the cells fixed to oxygen probes. Matsunaga et al. (1981) have shown that such a configuration is suitable for measuring the number of living cells. With increasing numbers of cells the current of the electrode decreases in correspondence to the increased respiration. The least detectable cell number was 10^6 /ml for bacteria and 10^5 /ml for yeasts.

A commercial device for assay of cell number, called 'Biocheck', has been developed by Higgins et al. (1987). It uses a mixture of different

redox mediators to detect a large number of relevant microbes via the redox systems of the cells. As few as 10^6 cells per ml can be determined within 2 min.

To establish the assimilation characteristics of microbes, the cells were fixed to an oxygen electrode and contacted with the substrates to be tested (Hikuma et al., 1980d). In contrast to conventional assimilation tests requiring cultivation of 24–72 h the sensor assay needed only 30 min. The physiological state of the cells can be determined in a similar manner (Riedel et al., 1985b).

Matsunaga and Nakajima (1985) succeeded in the differentiation between gram-negative and gram-positive bacteria by measuring current-potential curves of a carbon electrode bearing the fixed bacteria. Gram-positive cells gave rise to peak currents at 0.65–0.69 V vs SCE whereas the current peaks caused by gram-negative cells were found at 0.74 V. The cell number could be determined from the current change. The authors postulated that with *E. coli* and *Lactobacillus acidophilus* the peak currents were caused by electrochemical oxidation of coenzyme A. If this were the case, however, the current would not definitely correlate with the cell number because the formation of coenzyme A also depends on the physiological state of the cells.

3.3.3 Tissue Slices

Slices and other parts of tissues of animal or plant origin are the most complex biosystems so far applied in biosensors. Tissues containing large amounts of the enzymes of interest have been deliberately used. An overview of tissue-based sensors is given in Table 18.

Since tissue slices are easy to handle, they can be immobilized on electrodes by simple mechanical fixation using a semipermeable membrane or a nylon net. Additional crosslinking has been proposed in order to increase the mechanical stability (Kuriyama and Rechnitz, 1981).

At the University of Delaware one of the leading groups in tissue-based sensor research studied sensors using porcine kidney slices for the determination of glutamine (Rechnitz et al., 1979) and glucosamine-6-phosphate (Ma and Rechnitz, 1985). The tissue was attached to an ammonia gas-sensing probe. The glutamine sensor has been employed to measure glutamine in cerebrospinal fluid (Arnold and Rechnitz, 1980b). The selectivity for glutamine was increased by addition to the sample of iodoacetamide which inhibits the liberation of NH_3 by glycolysis.

TABLE 18

Tissue-Based Biosensors

Tissue (slice)	Substrate	Species detected	References
Beef liver (+urease)	arginine	NH ₃	cf. Rechnitz (1981)
Pig kidney	L-glutamine	NH ₃	Rechnitz et al. (1979); Arnold and Rechnitz (1980a,b)
Rabbit muscle	adenosine monophosphate	NH ₃	Arnold and Rechnitz (1981)
Yellow squash	L-glutamate	CO ₂	Kuriyama and Rechnitz (1981)
Beef liver	H ₂ O ₂	O ₂	Mascini et al. (1982); Mascini and Palleschi (1983b)
Rabbit liver	guanine	NH ₃	Arnold and Rechnitz (1982)
Sugar beet	tyrosine	O ₂	Schubert et al. (1983)
Corn kernel	pyruvate	CO ₂	Kuriyama et al. (1983)
Potato (+GOD)	phosphate; fluoride	O ₂	Schubert et al. (1984)
Cucumber leaf	cysteine	NH ₃	Smit and Rechnitz (1984)
Pig kidney	glucosamine-6- phosphate	NH ₃	Ma and Rechnitz (1985)
Mushroom	phenols	O ₂	Macholán and Schanel (1984)
Banana	dopamine	O ₂	Sidwell and Rechnitz (1985)
Squid nerve	di-isopropyl fluorophosphate	F ⁻	Uchiyama et al. (1987; 1988a)
Cucumber peel	ascorbic acid	O ₂	Vincké et al. (1985c); Macholán and Chmelikova (1986)
Banana peel	oxalate	CO ₂	Fonong (1986b)
Parts of carnation and chrysanth- emum flowers	amino acids, urea	NH ₃	Uchiyama and Rechnitz (1987)
Cabbage	sulfoxides	NH ₃	Sidwell and Rechnitz (1986)
Spinach leaf	catechol	O ₂	Uchiyama et al. (1988b)
Banana	dopamine	dopaquinone	Wang and Lin (1988)
Horseradish root	H ₂ O ₂	benzidine	Wang and Lin (1989)
Plant leaf	odor compounds	ion permeabil- ity	Matsuoka and Homma (1989)

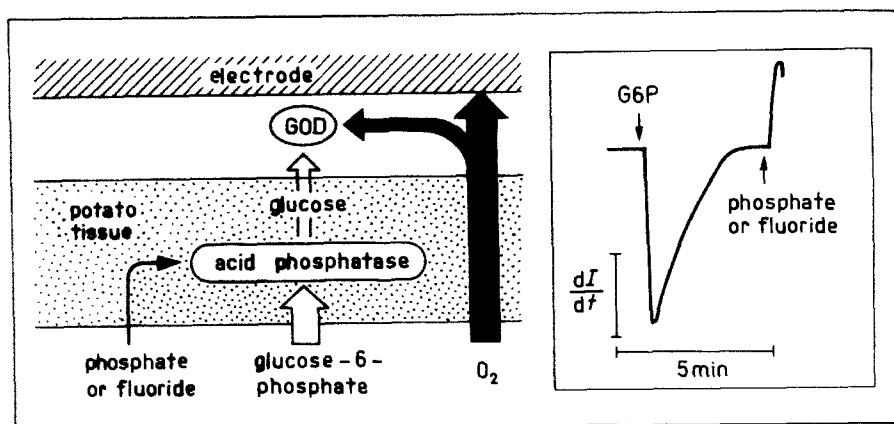


Fig. 111. Schematic representation and measuring curve of the determination of the inhibitors, phosphate and fluoride, with a GOD-tissue slice hybrid sensor.

In both the glutamine and the glucosamine-6-phosphate determination the substrate metabolism in the tissue gives rise to the formation of NH_3 which was indicated at the electrode. With both analytes present in the sample, selectivity for one of them could be achieved by a simple pH shift: the pH optimum for glutamine measurement was 8.5, that for glucosamine-6-phosphate 9.25. Although this difference is small, the optima are sharp enough to assure sufficient signal resolution. Another method to enhance the selectivity of HIS sensors was thus demonstrated.

Tissue-based sensors have also been designed using the hybrid sensor concept. Schubert et al. (1984) described a biosensor composed of glucose oxidase and potato tissue rich in acid phosphatase activity (Fig. 111). The sensor was suitable for the determination of the inhibitors of acid phosphatase, inorganic phosphate and fluoride. Glucose-6-phosphate present in the measuring solution was hydrolyzed by acid phosphatase, the glucose formed giving a stable base signal of the sensor. Addition of inhibitor resulted in a signal reflecting the diminished glucose liberation. This sensor benefits from the high stability of the acid phosphatase in its native environment. To obtain an inhibitor-dependent signal, the enzyme must not be present in excess, i.e., its concentration in the bioactive layer should be low. This means that the natural inactivation may not be compensated for by high activity, but must be minimized by optimal environmental conditions. Such conditions are present in the intact potato tissue. Therefore the sensor for phosphate and fluoride was stable for 4 weeks.

Sidwell and Rechnitz (1985) placed a slice of banana pulp tissue on the gas-permeable membrane of a Clark-type oxygen electrode. The banana tissue contains polyphenol oxidase which catalyzes the oxidation of dopamine to dopamine quinone and further to melanin at the expense of oxygen. Wang and Lin (1988) integrated this biocatalytic phase in the electrode body of a membrane-free carbon paste electrode and measured the formation of dopamine quinone at a potential of -0.2 V. This arrangement permitted selective determination of dopamine in the presence of ascorbic acid with a response time of only 12 s. It seems likely that this improved performance of a tissue-containing sensor could be extended to other analytes.

The capability of flowers and blossoms to convert organic compounds into volatile products has been utilized in biosensors using such plant tissues as biocatalytic material (Uchiyama and Rechnitz, 1987). Both minced and intact tissue portions from chrysanthemum and carnation flowers were coupled with potentiometric NH_3 electrodes to construct sensors for urea and amino acids. Surprisingly different selectivity patterns were found among species of flowers and for the structural subelements of a single type of flower. In particular, a sensor based on chrysanthemum receptacle tissue tested in a mixture of 20 amino acids was highly selective for L-asparagine, L-glutamine, and L-serine whilst chrysanthemum sepal electrodes, made from the same flower, showed a totally different selectivity pattern with a primary response to L-arginine, L-citrulline, and L-ornithine. Some of these sensors were active for several weeks.

Uchiyama et al. (1987, 1988a) immobilized squid nerve tissue in front of a fluoride ion sensitive electrode. The tissue contains diisopropyl fluorophosphatase, the activity of which was used to measure diisopropyl fluorophosphate. The sensor was stable for 18 days.

3.3.4 Miscellaneous Bioorganic Materials

Fitting into the trend towards improvement of the availability and simplification the preparation of biocatalytic layers for biosensors, the use of crude materials has been explored. Arnold and coworkers investigated the feasibility of employing Jack bean meal in a urea sensor (Arnold and Glaizer, 1984) and rabbit muscle acetone powder in a sensor for adenosine monophosphate (Fiocchi and Arnold, 1984). Both sensors turned out to be serious contenders with the appropriate enzyme electrodes with respect to lifetime and slope of the calibration curves. Other parameters, such as response time and linear range, were quite similar.

3.3.5 Lipid Membrane Biosensors

Jehring et al. (1979) studied the electrosorption of lecithin at dropping mercury electrodes. Compact monolayers were formed that closely resembled biological membranes. The permeation of ions, e.g. Cd^{2+} , could be specifically triggered by the coadsorption of acetylcholine and γ -aminobutyric acid. Other organic compounds were inactive. Furthermore, current oscillations somewhat analogous to the signal processing in nerve excitation were observed in the presence of acetylcholine.

A chemical sensor that mimics biological ion channels has been described by Umezawa et al. (1987). These authors covered carbon electrodes with Langmuir-Blodgett layers and introduced ionophores such as valinomycin. Addition of a stimulus, e.g. potassium ion, resulted in reversible opening of the channels. Ferricyanide marker ions could permeate through the lipid membrane to the underlying electrode resulting in a pronounced current signal.

Biomimetic membranes mimicking biological chemoreceptors have been formed by casting complexes of synthetic lipids and sodium polystyrenesulfonate onto a silicon wafer with a single pool (Hayashi et al., 1989). These membranes responded to negatively charged chemicals having strong bitterness. Obviously the measured potential change is due to nonspecific electrostatic interaction of the positively charged lipids with the anionic taste substances. Synthetic structures consisting of a planar bilayer of lipids, as described by Mueller (1962), are suitable for the study of interactions at or across cell membranes. The applicability of such bilayer lipid membranes (BLM) in biosensors has recently been examined (Krull and Thompson, 1985). In these sensors the measuring signal is generated by an ion flux resulting from potentials of 5–50 mV applied across the membrane. The ion flux is changed upon interaction of the analyte with the membrane. Such membranes have been made selective by the incorporation of a receptor. The formation of the analyte complex with the receptor changes the fluidity or ion permeability of the membrane, or gives rise to the formation of ion conductive pores. Promising results have been obtained with a BLM ammonia gas sensor incorporating an antibiotic to obtain selectivity (Thompson et al., 1983; Thompson, 1987). The interfacing of ordered lipid layers with stable substrates such as metal electrodes and hydrated gels is a precondition for reliable BLM sensors. One area with potential for such hybrid construction lies in the Langmuir-Blodgett thin film deposition technology which is presently being pursued for micro-device fabrication.

Chapter 4

Affinity Biosensors

As shown in Section 2.3, various biospecific recognition systems and interactions taking place *without analyte conversion* can be utilized for the construction of biosensors. When the binding of the analyte to an immobilized biomolecule or receptor system is reversible the sensor becomes reusable. Since the physicochemical changes caused by the binding are mostly very slight, in many cases auxiliary reactions have to be coupled.

The resulting affinity sensors can be composed of low-molecular weight biospecific ligands, proteins, enzymes, nucleic acids, and antibodies. Cell membrane components, cell organelles, and intact cells have also been employed. The latter approaches lead to immunosensors and rezeptrodes.

4.1 AFFINITY SENSORS USING LOW-MOLECULAR WEIGHT LIGANDS

Mandenius et al. (1986) proposed an optical biosensor for dehydrogenase assay based on the affinity of dehydrogenases for the coenzyme, NAD^+ . The sensor was assembled by covering a silanized silicon chip with dextran and subsequent covalent binding of NAD^+ to the dextran. The thickness of the fixed layer was determined with a refractometer from the polarization of light reflected by the layer. Addition of a sample containing alcohol dehydrogenase or LDH resulted in a change of the thickness due to the specific interaction of the dehydrogenase with bound NAD^+ (Fig. 112). The thickness change could be evaluated 30 s after sample injection. The chip was reusable. Regeneration was performed by washing with NAD^+ -pyrazole solution (2 mmol/l each) for binding of ADH in a ternary complex, or NAD^+ -oxalate solution (2 mmol/l each) for competitive binding of LDH. The chip was used in a flow-through device for ADH measurement. At a flow rate of 0.5 ml/min 1.7 μg ADH of a total of 0.1 mg/ml were bound per cm of the chip.

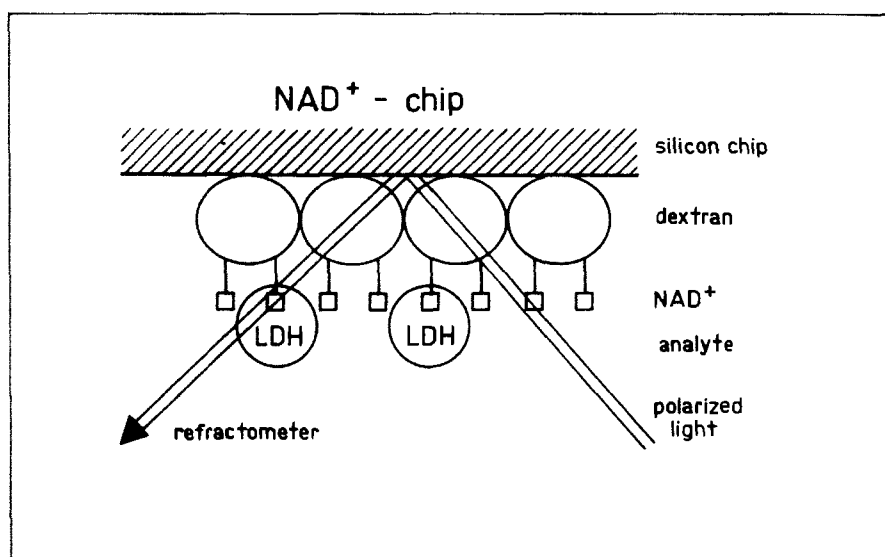


Fig. 112. Affinity sensor for dehydrogenases using refractometric indication. (Redrawn from Mandenius et al., 1986).

Goldfinch and Lowe (1980) developed a reagentless optoelectronic sensor for the determination of serum albumin by introducing a cellophane membrane bearing immobilized bromocresol green between a red light-emitting diode and a photodiode (see also Fig. 8). At pH 3.8 human serum albumin (HSA) was adsorbed on the immobilized dye, causing a color change from yellow to blue-green. The transmission of the red light was reduced accordingly. The sensor responded linearly to HSA concentrations in the range of 5 to 35 mg/ml. The binding of HSA was completely reversible and, with a CV of 1.4%, it was well reproducible. The sensitivity approached that of the common radioimmunoassay for HSA. The sensor is advantageous in that no radioactive material is required. Furthermore, the measuring time of only 15 s was significantly lower than that of other assay methods and the apparatus set-up was much less expensive.

4.2 AFFINITY SENSORS BASED ON PROTEINS AND ENZYMES

4.2.1 Binding Sensors

Many binding sensors make use of the unique, antibody-like properties of lectins. These plant proteins are capable of binding certain

carbohydrates or carbohydrate groups with high affinity and are therefore frequently employed for the specific purification of glycoproteins, e.g. blood group substances, by affinity chromatography.

One of the best-known lectins, concanavalin A (con A), obtained from Jack bean, selectively binds sugars. Janata (1975) was the first to explore the use of such binding behavior for chemical analysis (Table 19). He constructed an affinity electrode by binding con A to a PVC membrane in front of a platinum electrode. Mannan was chosen as a model analyte. The interaction of mannan with the immobilized lectin effects the potential across the electrochemical boundary layer at the PVC membrane. However, nonspecific binding of proteins causes similar effects. In order to eliminate these disturbances, another electrode with immobilized con A was used, but the con A was previously saturated with glucosamine and thus blocked against mannan binding. The difference between the signals of both electrodes reached an equilibrium value 30–45 min after addition of mannan. A relatively high detection limit of 0.1 mg/ml was obtained. Whereas the unspecific protein adsorption was compensated for, the problem of interferences by other carbohydrates bound to con A remained unresolved. Nevertheless, these investigations initiated the development of a multitude of immunoelectrodes.

Aizawa (1982) patented a lectin electrode based on the binding of horseradish peroxidase via its carbohydrate groups to con A immobilized on a hydrogen peroxide sensitive probe. The bound enzyme activity was determined from the H_2O_2 consumption catalyzed by HRP.

Con A has also been incorporated in an optical affinity sensor for glucose and other carbohydrates (Fig. 113) (Schultz and Sims, 1979). The sensor contained, at the tip of an optic fiber bundle, a reaction chamber with fluorescein-labeled dextran which was covered by a membrane permeable to low-molecular weight substances. Con A was immobilized on the inner wall of the chamber. The fluorescence was registered with a photodiode. The glucose determination was based on the competition of glucose with the labeled dextran for the binding to con A. As con A was immobilized outside the light path, the bound labeled dextran was inaccessible. The fluorescence of the labeled substance in the solution was therefore directly proportional to the concentration of glucose. The linear range was 1–28 mmol/l and the response time 5–10 min. The sensor was stable for several weeks. The sensitivity was limited by the large base signal. Nevertheless, this type of affinity sensor appears to be promising because it should also be useful for other analytes and permits continuous measurements.

TABLE 19

Affinity Sensors

Analyte	Immobilized species	Principle	Transducer	References
Mannan	con A	difference measurement between active and blocked con A electrode	Pt electrode	Janata (1975)
HRP	con A	specific binding and measurement of enzyme activity	H ₂ O ₂ electrode	Aizawa et al. (1982)
Glucose	con A	competition of glucose with fluorescein-dextran for con A	fiber optic sensor	Schultz and Sims (1979)
Glucose	con A	competition of glucose with fluorescein-glycogen for con A	fiber optic sensor	Srinivasan et al. (1986)
Glucose	con A	change of charge distribution in a column caused by specific analyte binding	electrode (streaming potential)	Mattiasson (1984)
Yeast cells	con A	change of layer thickness on the chip surface caused by analyte binding	refractometer, ellipsometer	Mandenius et al. (1984)
Biotin	HABA	competition of HABA with biotin for avidin-catalase	O ₂ electrode	Ikariyama et al. (1983)
B-Lymphocytes	protein A-catalase	decrease of catalase activity caused by binding of B-lymphocytes	O ₂ electrode	Aizawa (1983)
Formaldehyde	formaldehyde dehydrogenase	mass change caused by analyte binding	piezoelectric crystal	Guilbault and Ngeh-Ngwainbi (1987)
Malathion	acetylcholinesterase	mass change caused by analyte binding	piezoelectric crystal	Ngeh-Ngwainbi (1986b)

HABA = 2-(4'-hydroxyphenylazo)benzoic acid

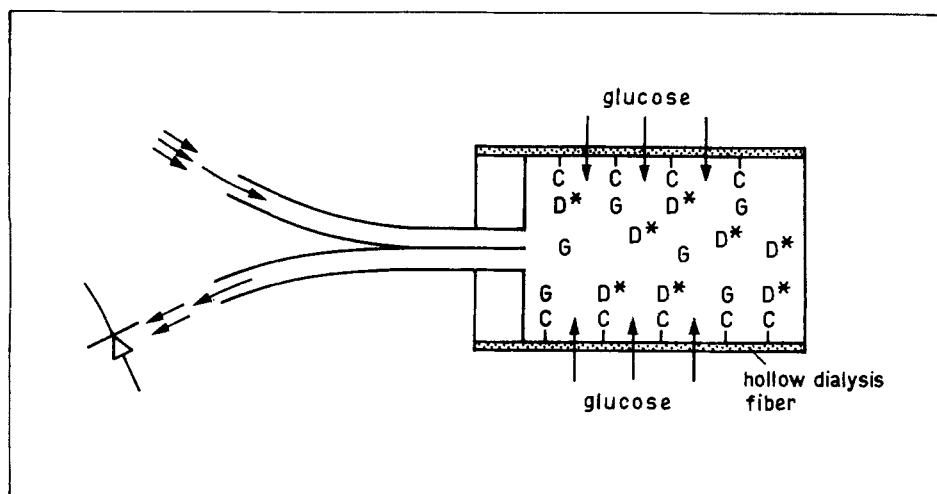


Fig. 113. Fiber optic affinity sensor for glucose and other low-molecular weight carbohydrates. C = immobilized con A, D* = fluorescein-labeled dextran, G = glucose.

In a more recent approach, the streaming potential in a miniature column containing immobilized biosorbent has been evaluated (Mattiasson, 1984; Glad et al., 1986). The interaction between the sorbent and the analyte could be indicated via the change of the charge distribution within the electrical double layer caused by the biospecific binding. Carbohydrates have been determined with this sensor system by using con A.

Another bioaffinity sensor (Table 17) makes use of the ability of avidin to bind biotin (vitamin H) as well as 2-(4'-hydroxyphenylazo)benzoic acid (HABA) (Ikariyama et al., 1983), the binding constant of avidin to HABA of $K = 1.7 \cdot 10^5$ l/mol being much lower than that to biotin of 10^{15} l/mol. The sensor can be used for biotin assay, because the avidin-HABA complex readily dissociates in the presence of biotin (Fig. 114). Avidin was labeled with catalase to increase the sensitivity for biotin. A cellulose triacetate membrane containing adsorbed HABA saturated with labeled avidin was attached to an oxygen electrode. The biotin to be determined was bound to the labeled avidin, resulting in a reduced catalase activity at the sensor. The remaining catalase was determined by addition of H_2O_2 and electrochemical indication of the liberated oxygen. The enzyme activity was inversely proportional to the concentration of biotin between $1 \cdot 10^{-9}$ and $5 \cdot 10^{-7}$ g/ml. This principle is also applicable to the assay of small molecules such as hormones and drugs.

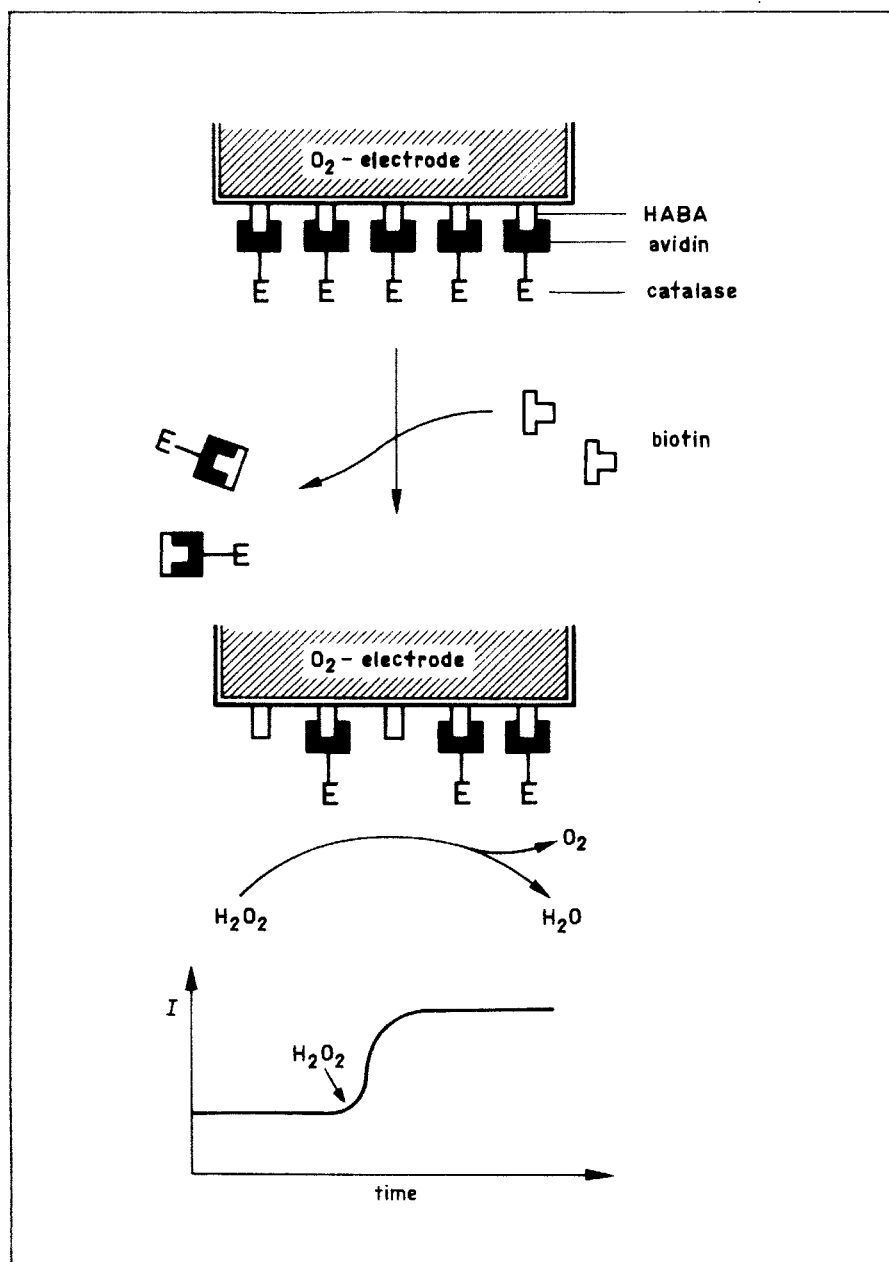


Fig. 114. Affinity electrode for determination of biotin.

Aizawa (1983) employed an oxygen electrode covered with protein A for the selective measurement of B-lymphocytes. As described above, the

activity of the marker enzyme, catalase, remaining after analyte binding was electrochemically indicated.

More recently, Guilbault and Ngeh-Ngwainbi (1987) constructed a reagentless sensor for formaldehyde by fixing crystalline formaldehyde dehydrogenase on the surface of a piezoelectric crystal. The measurements were carried out directly in the gas phase with only minor interference being encountered by other aldehydes and by alcohols. In a similar manner, gaseous organophosphate pesticides have been determined by using acetylcholine esterase attached to a piezoelectric crystal (Ngeh-Ngwainbi et al., 1986b). The calibration curve for malathion was linear up to 50 ppb with a response time of about 1 min and a relative standard deviation below 4%. In the dry state and at room temperature the sensor was stable for 40 days.

Fawcett et al (1988) developed a piezoelectric sensor device for monitoring the hybridization of complementary nucleic acid strands. The single stranded probe DNA was covalently attached to the polymer-modified surface of an ordinary piezoelectric crystal and melted by heat treatment. Hybridization with the target strand was carried out for 20 minutes at 4°C. After washing and drying the crystal, the resonance frequency relative to a control crystal was evaluated. The method requires very simple equipment and gives quantitative results for sample DNA of complementary nucleic acid sequences needed for colony hybridization in mixed culture samples. Regardless of the method used to detect the biomolecule–ligand interactions, all the assays described above suffer from insufficient selectivity. In many cases, no serious attempts were made to overcome this defect.

4.2.2 Apoenzyme Electrodes for the Determination of Prosthetic Groups

Generally, the binding of a prosthetic group to the apoenzyme is characterized by a high affinity and extraordinary specificity. In apoenzyme sensors the prosthetic group is recognized and bound by the apoenzyme, thus inducing enzyme activity which then serves to amplify the measuring signal. Under conditions of substrate saturation and apoenzyme excess the reaction rate is proportional to the concentration of the holoenzyme, i.e., to the amount of the prosthetic group to be analyzed.

This type of reaction is particularly sensitive when a soluble apoenzyme is used. FAD concentrations as low as 10^{-12} mol/l have been measured by using dissolved apo-GOD and electrochemical indication

of the H_2O_2 formed in the GOD reaction (Ngo and Lenhoff, 1980). The vitamins B_6 (pyridoxalphosphate, PLP) and B_1 (thiamine pyrophosphate) have been assayed in a similar manner by using apo-tyrosine decarboxylase (Hassan and Rechnitz, 1981) and apo-pyruvate decarboxylase (Seegopaul and Rechnitz, 1983). Under optimal conditions a PLP concentration of 1 nmol/l could be detected with an amplification of 10^5 . Marker enzymes, e.g. alkaline phosphatase, can be determined by using an inactive derivative of a prosthetic group, e.g. phosphoric acid esters of PQQ, and the respective apoenzyme, e.g. apo-glucose dehydrogenase (PQQ). The marker enzyme reaction forms the active prosthetic group which is subsequently bound to the apoenzyme, thus leading to a cascade-like substrate conversion. This principle has also been used in DNA hybridization tests (Higgins et al., 1987).

An apoenzyme electrode for Cu^{2+} has been developed by the coupling of immobilized apo-tyrosinase with an oxygen probe (Mattiasson et al., 1979). The detection limit of the sensor was 50 ppm. However, the reusability of such an apoenzyme membrane appears to be questionable because the enzyme activity accumulates during the operation of the sensor whereas the measuring principle requires kinetic control so as to obtain a linear dependence of the sensitivity on the activity of the holoenzyme.

Jasaitis et al. (1983) proposed a carbon electrode with covalently bound alkaline phosphatase (AP) for the determination of Zn^{2+} . Apophosphatase was created by treating the electrode with EDTA. Addition of a zinc ion-containing sample restored the enzymatic activity of AP within 30 s as demonstrated by the formation of electrode-active hydroquinone from hydroxyphenyl phosphate. After each measurement the electrode was regenerated by treatment with EDTA. As little as 0.8 $\mu\text{mol/l}$ of Zn^{2+} could be detected.

4.2.3 Enzyme Sensors for Inhibitors

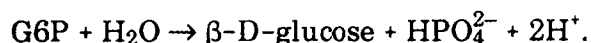
Biosensors for inhibitor determination are based on the ability of inhibiting substances to become bound to the receptor component and decelerate the substrate conversion. Therefore, inhibitor sensors, similar to apoenzyme sensors and immunosensors, combine the affinity principle with enzymatic amplification reactions. In contrast to metabolism sensors, the binding is evaluated rather than the chemical reaction of the analyte.

The limitation of the overall process by the rate of the enzyme reaction, i.e., kinetic control, is an important precondition for inhibitor

assay with biosensors. It is given at low enzyme loading factor or substrate saturation. While some enzymes are inhibited only by a narrow range of special substances, others, such as acetylcholine esterase, are sensitive to whole classes of substances. Being affected by various toxic agents, the metabolism of microorganisms can be used as a sensitive indicator of toxicity (see Section 3.3.2).

In the case of competitive inhibition the substrate and the inhibitor compete for the enzyme binding site. The same is true for product inhibition, where the accumulation of a product leads to a slow down of the enzyme reaction. Prominent examples are the inhibition of AP by phosphate, of arylsulfatase by sulfate, and of cholesterol oxidase by cholestenone.

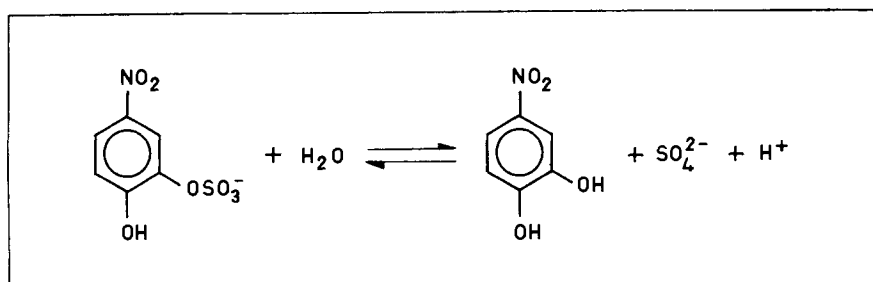
An enzyme sequence electrode for phosphate assay based on AP and GOD has been devised by Guilbault and Nanjo (1975b). Glucose-6-phosphate (G6P) was used as the substrate for AP:



Addition of phosphate diminishes the rate of glucose formation, so that less hydrogen peroxide is formed in the GOD-catalyzed sequential reaction. Consequently, the hydrogen peroxide oxidation current of the sensor decreases. Interferences were found to occur by arsenate and tungstate which also inhibit the AP reaction.

For the assay of maltitol, Renneberg (1988) coupled the competitive inhibition by maltitol of the glucoamylase-catalyzed maltose hydrolysis with the GOD-mediated indication of the liberated glucose.

Cserfalvi and Guilbault (1976) developed a sensor for the measurement of sulfate by using arylsulfatase (EC 3.1.6.1). Arylsulfatase catalyzes the hydrolysis of arylsulfates under formation of an electrode-active product:



The semilogarithmic plot of the sensor signal versus sulfate concentration showed that the inhibition was linearly dependent on pSO_4^{2-} be-

tween 0.1 and 10 mmol/l. The slope of the calibration curve increased with decreasing enzyme loading, indicating the importance of kinetic control for the determination of inhibitors.

In a sensor for cholestenone based on immobilized cholesterol oxidase, Wollenberger et al. (1983) found an increase of the inhibitor constant to 2.2 mmol/l as compared with 0.13 mmol/l for the free enzyme. The steady state current of the sensor depended nonlinearly on cholestenone concentration. The maximum inhibition was 50%.

Albery et al. (1987a) developed a carbon monoxide sensor based on the sequence of cytochrome oxidase and cytochrome c coupled to a modified gold electrode. The inhibition by CO was detected via the decrease of the oxygen reduction rate. The sensor is also applicable to the quantitation of other inhibitors of the respiratory chain.

The inhibitory action of Hg^{2+} , Pb^{2+} , Zn^{2+} , and other heavy metal ions on SH-containing enzymes has been utilized to detect heavy metal ions by using immobilized GOD (Liu et al., 1982). Since the inhibition is irreversible, the enzyme can only be applied to the assay of one sample.

Organophosphorus compounds are irreversible inhibitors of acetylcholine esterase and butyrylcholine esterase (BuChE, EC 3.1.1.8) because the phosphate group is irreversibly bound by the enzyme. Therefore, organophosphorus pesticides can be detected by using the free enzyme. Since the activity of cholinesterases (ChE) in normal serum is rather large (800 U/l), untreated serum pools may be employed for inhibitor determination. Gruss and Scheller (1987) have shown that the hydrolysis of butyrylthiocholine iodide can be directly indicated at a membrane-covered platinum electrode polarized to +470 mV. Twenty seconds after sample addition a steady value proportional to the enzyme activity was obtained in the differentiated current–time curve. Injection of an inhibitor decreased the rate of thiocholine formation, so that the residual activity could be evaluated after 30 s (Fig. 115).

Razumas et al. (1981) employed indoxylphosphate as the substrate for ChE and registered the formation of indigo at +300 mV. By pre-incubating the samples with 0.4 U of ChE for 3–10 min, the authors were able to detect insecticides in the range 10–600 pmol/l.

Tran-Minh et al. (1986) fixed BuChE in a film of 10 μm thickness around the active tip of a pH glass electrode and measured the pH decrease during substrate hydrolysis. The electrode was inserted into a flow-through cell. When a steady pH value in 5 mmol/l butyrylthiocholine solution was reached, a competitive inhibitor, e.g. carbamate, was injected. The inhibition of the hydrolytic reaction resulted in an in-

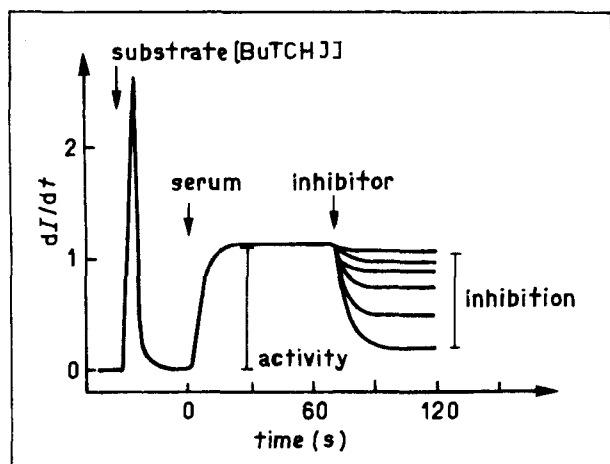


Fig. 115. Determination of cholinesterase activity and inhibitors with the Glukometer. BuTCHI = butyrylthiocholine iodide. (Redrawn from Gruss and Scheller, 1987).

creased pH value in the enzyme layer. After each assay the enzyme was regenerated with pyridine aldoxime.

This principle has been expanded to a dual electrode arrangement in which pH differences in the samples could be compensated (Durand et al., 1984). With a BuChE loading of 7.5 U/cm and under substrate saturation conditions the system was sensitive to micromolar inhibitor concentrations. The inhibition was markedly different with different pesticides. Such sensors are superior to physicochemical assays in that they detect the effectiveness of the inhibition.

The reversible inhibition of urease has been used to measure Hg^{2+} in the concentration range 0–150 nmol/l (Ögren and Johansson, 1978). Urease was immobilized on porous glass and packed into a reactor of 14 μl volume. A urea solution was pumped through the reactor, the urea concentration being so large that only 3% was converted in the reactor. The decrease of the formation of ammonia was monitored by means of a glass electrode. The inhibition depended linearly on the amount of Hg^{2+} pumped through the reactor. When the enzyme reactor was regenerated with thioacetamide and EDTA, it was stable for several measuring cycles. From quantitative studies the authors concluded that the inactivation of urease requires one or two Hg^{2+} -ions per enzyme subunit.

Tran-Minh and Beaux (1979) investigated the competitive inhibition by fluoride of urease bound to the silicone rubber membrane of a carbon

dioxide-sensing electrode by glutaraldehyde. A linear dependence of the potential on $\log[F^-]$ was obtained between 0.5 and 10 mmol/l. Studies with varying enzyme loading led to the following observations: whereas the urea calibration curves were essentially the same for different urease activities, which indicates diffusion control, the relative inhibition markedly depended on the enzyme loading. With decreasing loading the fluoride calibration curve was shifted to lower fluoride concentrations (Fig. 116). This means that the excess enzyme was 'titrated' by the inhibitor even when the rate of the urea hydrolysis was independent of the amount of enzyme. This effect has been confirmed by using sensors with the enzyme immobilized by a varying degree of glutaraldehyde crosslinking. The longer the enzyme was in contact with glutaraldehyde, the lower was the remaining activity and the higher was the sensitivity for fluoride. In essence, these findings once again indicated that in biosensors for substrate determination a large enzyme excess is required in order to minimize disturbances by inhibitors.

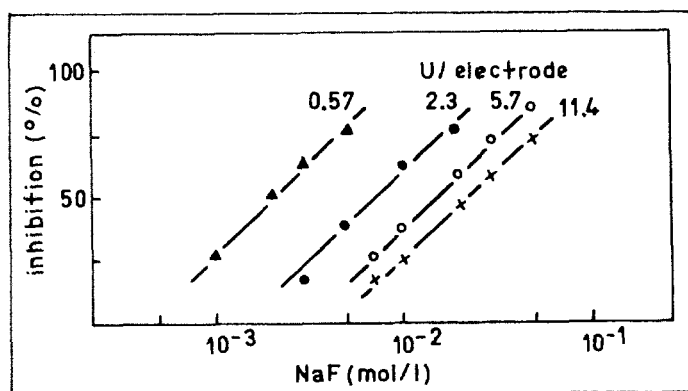


Fig. 116. Inhibition of urease as it depends on the concentration of fluoride and the enzyme loading of the sensor. (Redrawn from Tran-Minh and Beaux, 1979).

4.3 IMMUNOSENSORS

4.3.1 Principles of Immunoassays

Antibodies reversibly bind antigens or haptens with affinity constants between $5 \cdot 10^4$ and 10^{12} l/mol. The complex formation is difficult to measure because it proceeds much more slowly than the reactions of low-molecular weight compounds. Sensitive methods for monitoring

immunological reactions and thus measuring one of the reaction partners are based on the labeling of one of the immunoreactants.

Whereas in homogeneous, separation-free immunoassays the activity of a marker bound to antigen is affected by the binding of antibody, in heterogeneous immunoassays this activity remains unaffected by the immunological reaction. Therefore, in heterogeneous immunoassays, the free conjugate must be separated from the immunoreagent-bound conjugate before the marker activity can be measured. For the measurement of antigens, competitive binding assays using labeled antigens, and sandwich assays using labeled antibodies are most often performed (Fig. 117).

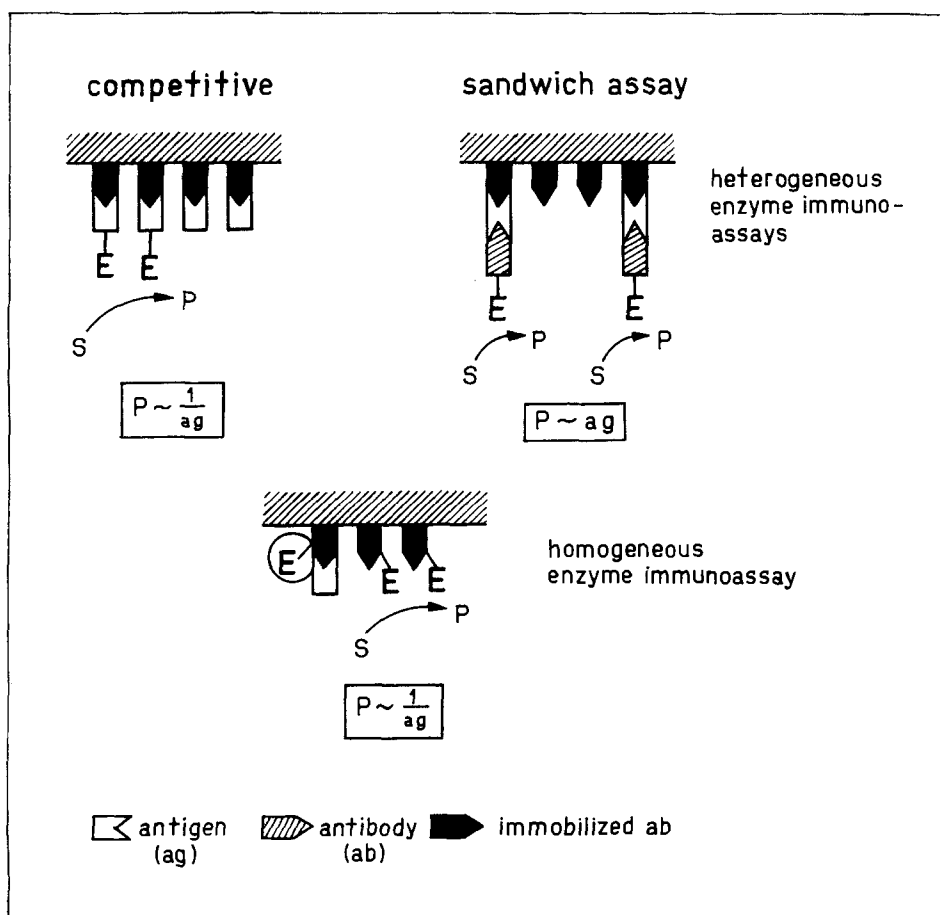


Fig. 117. Principles of enzyme immunoassays for antigen determination.

The introduction of the radioimmunoassay (RIA) technique by Yalow and Berson (1959) enabled the specific determination of very small amounts of substances. This method combines the specific molecular recognition during immunochemical interactions with the extremely high sensitivity of isotope detection and has therefore gained general acceptance. In competitive RIA non-labeled antigen competes with labeled antigen for the binding sites of a known amount of complementary antibody. After removal of excess antigen the radioactivity of the antigen-antibody complex is inversely proportional to the antigen concentration in the sample.

In order to avoid the drawbacks of RIA, particularly the potential hazards from radioactive material, other indicators have been introduced, among them enzymes, fluorophores, and luminescence markers. Enzymes permit rather sensitive measurements of immunological reactions by providing a catalytic signal amplification. Therefore, enzyme immunoassays (EIA) are an attractive alternative to RIA. In contrast to isotopes, enzymes can also be used in homogeneous, separation-free assays and their reactions can be monitored with simple devices (Tijssen, 1985). The sensitivity of RIA lies generally between 1 and 500 pmol/l. A comparable sensitivity has been described for heterogeneous EIA for HBs-antigen and insulin. Most other EIA are less sensitive (Oellerich, 1980).

Two directions of immunosensor development can be distinguished: direct sensors register electrochemical, optical, or electrical changes at the transducer surface resulting from the immunochemical complex formation. Similar to homogeneous and heterogeneous immunoassays, indirect methods are based on the labeling of one of the immunochemical reaction partners. Enzyme immunosensors belong to the latter class. They combine the selective immunocomplex formation with the high sensitivity provided by enzyme-chemical amplification.

4.3.2 Electrode-Based Enzyme Immunoassays

A problem of great importance to the development of immunoassays is their adaption to electrochemical detection processes (Ngo, 1987). Direct polarographic monitoring of immunological reactions has been shown to be possible by measuring the Brdicka-current of albumin in the presence of cobalt salts and anti-albumin antibody (Alam and Christian, 1984) and by differential pulse polarography of estrogen antibody in solutions containing dinitroestriol (Wehmeyer et al., 1982).

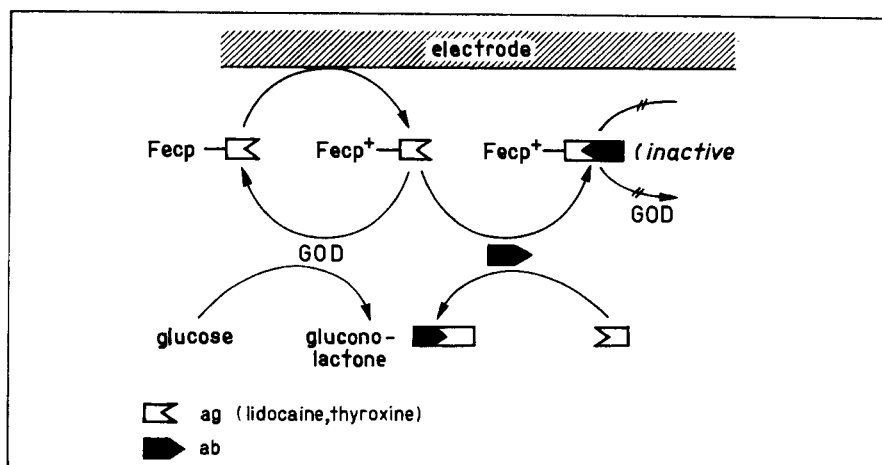


Fig. 118. Homogeneous electrode-based enzyme immunoassay. Fecp = ferrocene.

In homogeneous competitive assays the electrode-active group of the labeled antigen is masked by the bound antibody. Such electrochemical immunoassays have been developed for human serum albumin (HSA) (Alam and Christian, 1982, 1985). HSA was labeled with Pd^{2+} or Zn^{2+} , and the bound metal ion was measured by differential pulse polarography at a mercury electrode. Binding of antibody caused a drop of the peak current.

Other authors used ferrocene as a marker to measure morphine at a glassy carbon electrode (Weber and Purdy, 1979) or labeled HSA with metal chelates followed by voltammetric measurement of indium ions liberated in the immunological reaction (Doyle et al., 1982).

The sensitivity of electrochemical processes can be increased by coupling them with chemical amplification as provided by enzymatic catalysis. Di Gleria et al. (1986) and Robinson et al. (1986a) employed ferrocene as an electron acceptor for the GOD reaction to design homogeneous electrode-based immunoassays for lidocaine and thyroxine (Fig. 118). Ferrocene was conjugated with the appropriate antigen. The conjugate is coenzymatically active with GOD. When antibody is bound to the conjugate, this activity is blocked and consequently no ferrocene is reduced in the presence of GOD and glucose, and no catalytic current of ferrocene reoxidation is observed. The antigen to be determined then competes with the conjugated antigen for the bound antibody and thus reverses the blocking. In this way the measured current is directly proportional to the analyte concentration.

For thyroxine measurement linearity was obtained between 25 and 400 nmol/l, for lidocaine between 5 and 50 $\mu\text{mol/l}$, i.e., in the clinically relevant range. Lidocaine has been determined in plasma samples with a measuring time of 15 min per sample. The method is also applicable to other haptens such as digitoxin, theophylline, and phenobarbital. Increasing the sensitivity up to that of conventional RIA should make this kind of assay of practical utility.

Ngo et al. (1985) described the use of apo-GOD conjugated with 2,4-dinitrophenol (DNP) for DNP-aminocaproic acid measurement in a competitive immunoassay. The reconstitution of the activity of GOD from DNP-apo-GOD and the prosthetic group, FAD, was monitored amperometrically via the formation of hydrogen peroxide in the presence of glucose. The principle of the assay is depicted in Fig. 119. When DNP antibody is bound to the apoenzyme-DNP conjugate, the GOD activity cannot be reconstituted by addition of FAD. In the presence of the analyte, DNP-aminocaproic acid, most of the antibody is bound to the analyte. Therefore only a small amount of the inactive antibody-DNP-apo-GOD complex is formed. In the presence of glucose and FAD, H_2O_2 is produced by the reconstituted holoenzyme. The electrochemical oxidation current of H_2O_2 is proportional to the concentration of antibody.

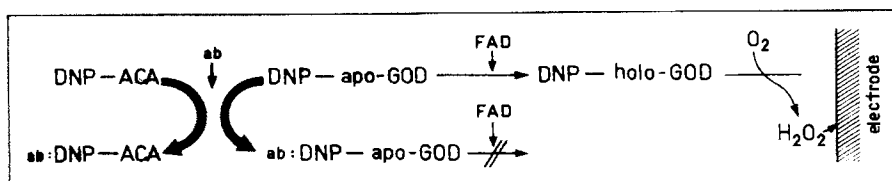


Fig. 119. Homogeneous electrode-based EIA for DNP-aminocaproic acid (DNP-ACA).

Most EIA use HRP as the marker enzyme. The activity of HRP can be measured photometrically as well as electrochemically. Using the catalase activity of liver tissue, Mascini and Palleschi (1983b) developed a tissue-based electrode for the measurement of hydrogen peroxide and combined the sensor with commercial test kits for digoxin and insulin. The HRP-labeled hormones of the test kit compete with antigen in the sample in a test tube. The bound HRP activity is inversely proportional to the concentration of insulin and digoxine, respectively.

The inhibition of the catalytic activity of chloroperoxidase (Cl-POD)

by the binding of antigen to a Cl-POD-antibody conjugate has been employed in a homogeneous potentiometric EIA for human immunoglobulin G (IgG) (Fonong and Rechnitz, 1984b). The catalytic formation of CO₂ from β -ketoadipinate was monitored with a carbon dioxide-sensing probe. The sensitivity of this system was much lower than that of RIA.

Glucose-6-phosphate dehydrogenase has been used as a marker enzyme in a heterogeneous EIA for phenytoin (Eggers et al., 1982). The formation of NADH in the dehydrogenase-catalyzed reaction was measured amperometrically. In order to achieve a rapid analysis, the FIA technique was used. A complete measuring cycle took at most 8 min. The current was linearly dependent on phenytoin concentration in the range 1–30 μ g/ml. Electrode fouling by sample proteins was prevented by cycling the electrode potential between +1.5 and –1.5 V between measurements.

A heterogeneous EIA coupled with a potentiometric electrode permitted the assay of BSA down to 10 ng/ml and cAMP down to 10 nmol/l (Meyerhoff and Rechnitz, 1979). Urease was used as the marker enzyme and its activity was measured by means of an ammonia gas-sensing electrode. The equilibrium of the immunological reaction at the sensor was reached rather slowly. The advantage of the rapid response of biosensors could not therefore be exploited.

Renneberg et al. (1983a) described an enzyme electrode-based assay of factor VIII, which is important for blood coagulation diagnostics. AP was used as the marker enzyme and the hydrolysis of glucose-6-phosphate was measured with a glucose electrode. This combination allowed the determination of 1.6–16 ng of factor VIII in human plasma.

In competitive heterogeneous EIA for digoxine (Wehmeyer et al., 1986) and human orosomucoid (Doyle et al., 1984), alkaline phosphatase was conjugated with the appropriate antigen. The enzyme activity was determined by using phenylphosphate as the substrate. The enzymatically liberated phenol was oxidized at a carbon paste electrode after chromatographic separation. As little as 1 ng/ml orosomucoid and 50 pg/ml digoxine could be detected. Since the detection of phenol was not limiting in the overall process, still lower detection limits might be achieved by using antibodies with higher binding constants.

Another way to improve this procedure would be the employment of modified mediators that could be split enzymatically to electrode-active products. The sensitivity has been increased and the measuring range expanded by using a ferrocene derivative, N-ferrocenoyl-4-amino-

phenylphosphate (Higgins et al., 1987). AP bound to the immunological complex hydrolyzes the modified mediator to phenylphosphate and ferrocene which is indicated electrochemically. In this manner, $8 \cdot 10^{-16}$ mol AP has been detected within 15 min. The method has been applied to the measurement of estriol.

Stanley et al. (1985) combined the advantages of mediators and enzymatic amplification in immunoassays by designing a reaction cycle of the marker enzyme AP with the use of NADP^+ as substrate (Fig. 120). NADP^+ is dephosphorylated by AP. The NAD^+ formed is reduced in the presence of ADH to NADH which, in the presence of diaphorase, shuttles reducing equivalents to ferrocene. The NAD^+ liberated in the latter reaction can enter the cycle again. Such an amplification system can also be applied to DNA hybridization tests (Downs et al., 1987).

As an alternative to the catalytic activity of a conjugated enzyme, the complement-mediated immunolysis of liposomes can be used to amplify the measuring signals of immunoassays. The liberation of liposome-entrapped markers depends on the amount of antibody adhering to the lipid membrane and serves to measure the concentration of antigen. Such liposomes may be filled with quarternary ammonium ions detectable by ion sensitive electrodes (Umezawa et al., 1983), with enzyme substrates, e.g. glucose, which can be measured with enzyme electrodes

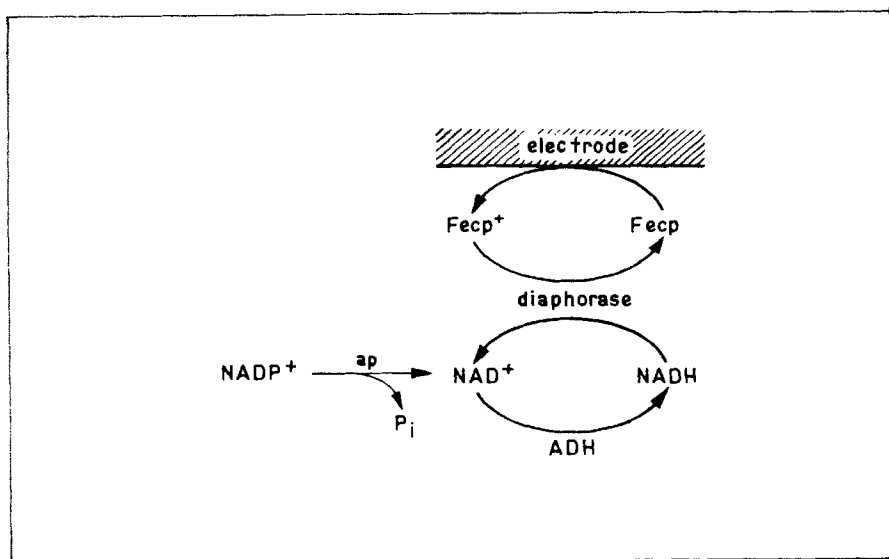


Fig. 120. Determination of the activity of the marker enzyme, alkaline phosphatase (AP), using enzymatic amplification.

(Umezawa et al., 1982), or with fluorescence markers (Ishimori et al., 1984). The latter system was shown to be most sensitive, the detection limit being 10^{-15} mol/l. Double signal amplification can be obtained by inclusion of enzymes in the liposomes (Brahman et al., 1984). Thus, Haga et al. (1980) entrapped HRP in sensitized liposomes and used the liposomes to determine theophylline. The rate of HRP liberation was monitored by measuring the NADH oxidase activity of HRP with an oxygen electrode. The electrode response correlated with the theophylline concentration in the sample between 4 and 20 nmol/l.

In summary, it can be stated that methods of electrochemical detection are very applicable to enzyme immunoassays. Broadly speaking, the above examples demonstrate that homogeneous EIA are faster and simpler but often less sensitive and more subject to interference than heterogeneous EIA. The latter are less sensitive to interference and electrode fouling because the measuring chamber in front of the electrode is rinsed before determination of the marker activity. However, none of the methods described is suitable for continuous measurement.

The determination of antigens and antibodies can be improved by immobilization of the appropriate immunochemical partner of the analyte. When the immunological reaction can be rendered reversible the immobilized ligand becomes reusable. Whereas in immunoreactors the immunosorbent is separated from the sensor, in immunosensors both elements are in intimate physical contact. We shall show below that these devices can be used to perform the classical functions of immunoassays.

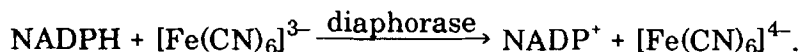
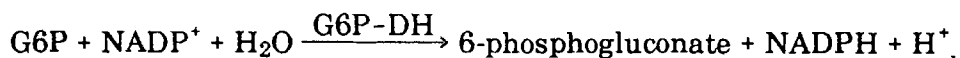
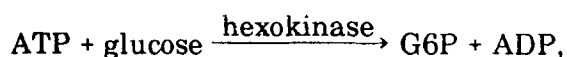
4.3.3 Immunoreactors

4.3.3.1 Immunoreactors with Electrochemical Detection

An immunoreactor with electrochemical detection based on the sandwich test has been developed by De Alwis and Wilson (1985). A minireactor was packed with Reactigel-6X containing immobilized IgG and introduced into a flow-through device. At a flow rate of 0.5 ml/min the sample (mouse-anti-bovine IgG antibody) and, after two minutes, the labeled analyte (anti-mouse-IgG-antibody-GOD conjugate) was injected. The activity of GOD and thus the amount of anti-IgG antibody was measured by amperometric hydrogen peroxide oxidation after three successive glucose injections. After each assay the reactor was regenerated by elution of the conjugate with a buffer of pH 2.0. Equilibration for the next measurement needed 10 min. The system was sensitive

for femtomolar to picomolar analyte concentrations with a CV of about 3%. Higher antibody concentrations could be detected by decreasing the incubation time to at least 6 s. The reactor was stable for 3 months or 500 measurements before the immobilized IgG lost its reactivity. Continuing this development, the authors succeeded in detecting subpicomolar concentrations of IgG by using Fab-fragments of human anti-IgG antibody (De Alwis and Wilson, 1987). A measuring time as low as 12 min was obtained by combining the reactor with an FIA manifold with electrochemical detection.

An immunostirrer for the determination of creatine kinase (CK) isoenzyme MB based on alkylamine glass-immobilized anti-IgG antibody has been proposed by Yuan et al. (1981). By binding of creatine kinase to antibody, only the CK-M subunit but not the CK-B subunit is inhibited. The remaining CK-B activity was measured by electrochemical oxidation of ferrocyanide formed in the following coupled reaction:



With a maximum binding capacity for CK-M of 800 U/l and a CV of 4–5% the system was stable for 52 days.

Aizawa et al. (1979b, 1980a) measured the change in the transmembrane potential across a cardiolipin-coated membrane (Fig. 121) resulting from antibody binding to assay Wassermann antibody and blood groups. An acetylcellulose membrane containing immobilized Wassermann antibody or human blood group substances, respectively, was used to separate two electrochemical half cells. After injection of the sample into one of the compartments, the antibody–antigen complex formation results in a shift of the potential drop in the double layer between the membrane and the measuring solution. The concentration of antibody was determined from the potential difference of two reference electrodes. The method appears to be not overly reliable because substances not

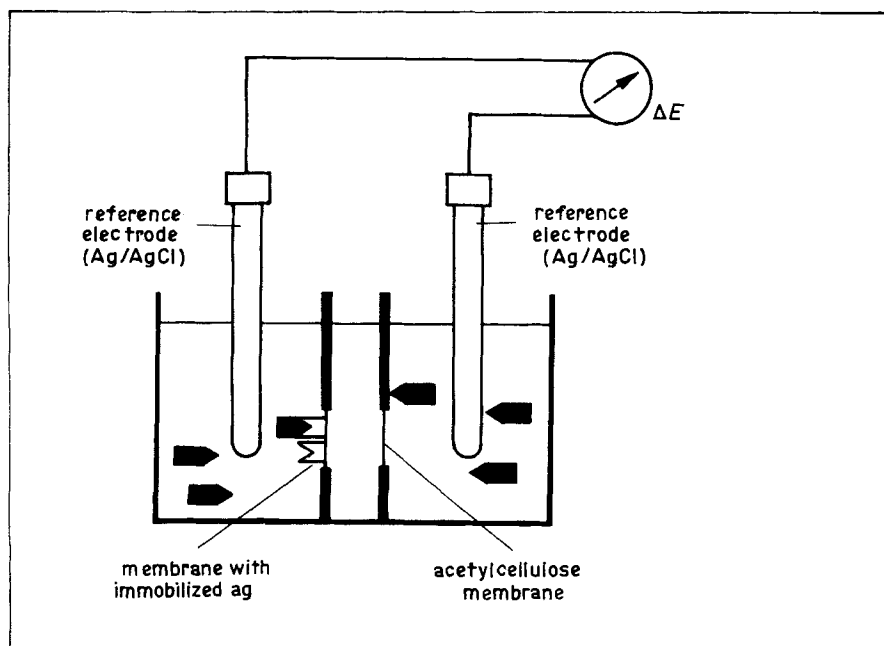


Fig. 121. Determination of Wassermann antibody in the syphilis test by measurement of the transmembrane potential change (ΔE). (Redrawn from Aizawa et al., 1977).

correlating with the analyte concentration can also cause potential shifts; accordingly, the reproducibility of the measuring signals was poor.

4.3.3.2 Thermometric Enzyme Immunoassays

Thermometric enzyme immunoassays (TELISA) are heterogeneous enzyme immunoassays with calorimetric indication. They involve the use of flow-through reactors incorporating antibodies covalently bound to a solid phase such as Sepharose CL-4B. After the immunological reaction of non-labeled antigen in competition with enzyme-labeled antigen the activity of the marker enzyme is determined by using a thermistor. The application of flow-through devices permits the measurement to be carried out under non-equilibrium conditions (Borrebaeck et al., 1978; Birnbaum et al., 1986) and thus reduces the measuring time from several hours to a few minutes. The course of such measurements with time is outlined in Fig. 122. TELISA have been employed for the fast determination of proinsulin (Birnbaum et al., 1986), gentamicin, and albumin (Borrebaeck et al. 1978) (Table 20). The antigen can be

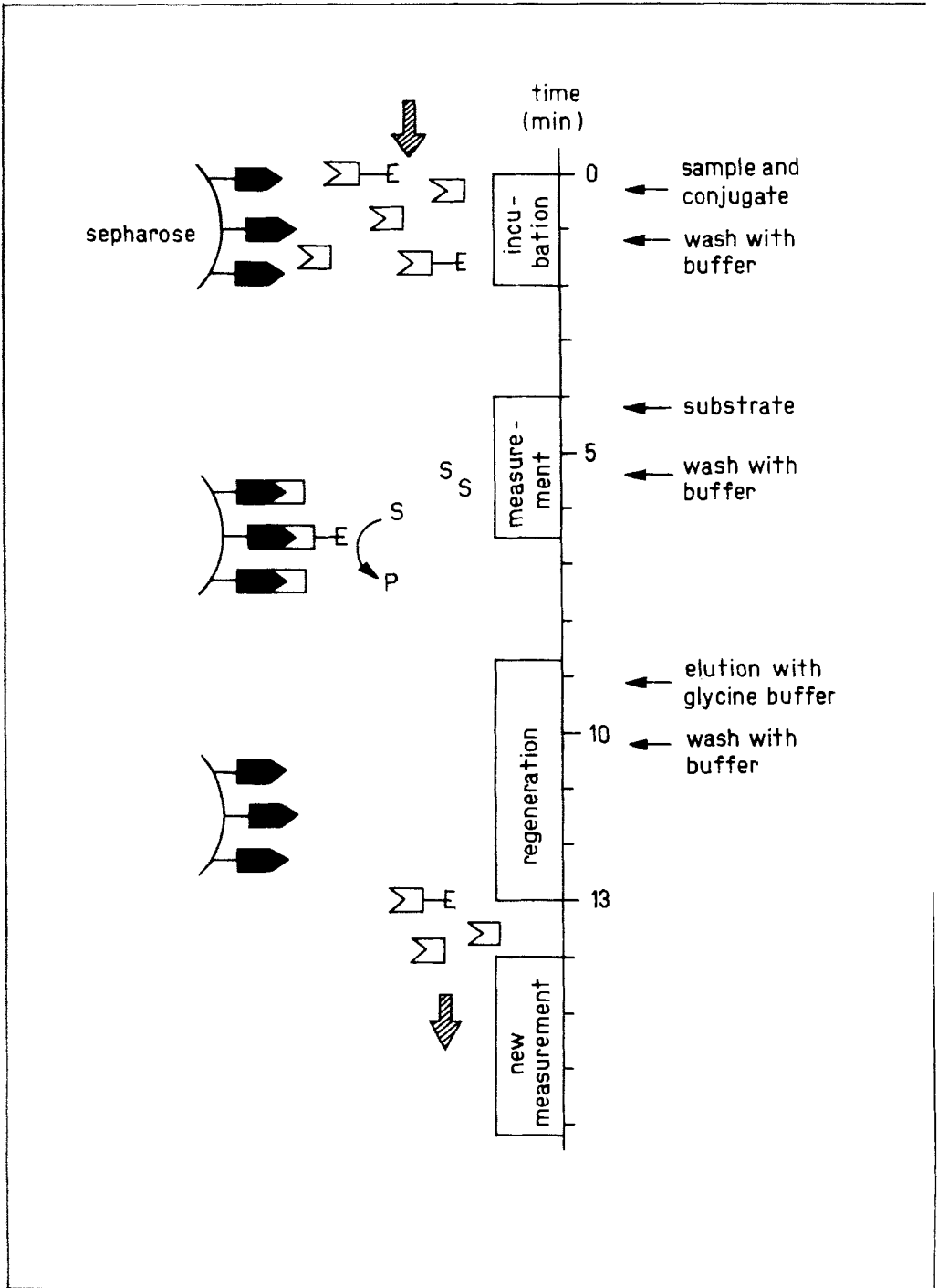


Fig. 122. Principle and time course of TELISA.

removed from immobilized antibody by washing the complex with 0.2 mol/l glycine-HCl buffer, pH 2.2. In this way the carrier is regenerated. The sensitivity of TELISA is far below that of RIA or fluorescence immunoassays.

TABLE 20

Thermometric Enzyme Immunoassays Using Antibody Covalently Bound to Sepharose CL-4B

Analyte	Immuno sorbent	Marker enzyme	Measuring range	Measuring time (min)	Lifetime	References
Genta-micin	anti-gentamicin antibody	catalase HRP	0.01–0.9 $\mu\text{g/ml}$	9–12	8 days	Borrebaeck et al. (1978)
HSA	anti-HSA antibody	catalase	$1 \cdot 10^{-13}$ – $1 \cdot 10^{-8}$ mol/l		15–20 det.	Borrebaeck et al. (1978)
Pro-insulin	anti-insulin antibody	HRP	$1 \cdot 10^{-8}$ – $5.5 \cdot 10^{-6}$ mol/l	13 min	14–21 days	Birnbaum et al. (1986)

The flow-through TELISA has been automated and used to monitor the production of human proinsulin by genetically manipulated *Escherichia coli* cells. Good agreement with the results of an RIA was obtained. The short analysis time, reusability, and automatability of the flow-through TELISA technique offer further prospects for application in fermentation control and clinical chemistry.

4.3.4 Membrane Immunosensors

Enzyme immunoelectrodes involve the spatial coupling of the sensor, the immunocomplex, and the catalytic amplification by indicator enzymes. Like the sensor systems described above, enzyme immunoelectrodes are based on common principles of EIA. The choice of enzymes for EIA is rather restricted and is further diminished when electrodes are to be used for detection. So far only GOD, catalase, and HRP have been combined with oxygen-sensing polarographic sensors. An overview of enzyme immunoelectrodes is given in Table 21.

In one of the first reports concerning amperometric immunosensors, Mattiasson and Nilsson (1977) proposed an electrode system for determining insulin and albumin. An oxygen electrode was covered by a nylon

net carrying a fixed antibody and inserted into the measuring chamber of a flow-through analysis system. The measurement was conducted according to the principle of competitive EIA. The marker enzymes used were catalase for insulin assay and GOD for albumin assay. For measurement, the sample was contacted with the membrane in the presence of a known amount of labeled antigen. In contrast to conventional EIA

TABLE 21

Enzyme Immuno-electrodes for Antigen Determination

Antigen	Electrode	Principle	Marker enzyme	Sensitivity	References
Theophylline	O ₂	competitive	catalase	$1 \cdot 10^{-3}$ – $5 \cdot 10^{-3}$ mol/l	Haga et al. (1984)
	O ₂	sandwich	catalase	$5 \cdot 10^{-8}$ – $2.5 \cdot 10^{-6}$ mol/l	Shimura et al. (1986)
Insulin	O ₂	competitive	catalase	10^{-6} mol/l	Mattiasson and Nilsson (1977)
AFP	O ₂	competitive	catalase	$5 \cdot 10^{-11}$ – $1 \cdot 10^{-8}$ g/ml	Aizawa et al. (1980b)
HCG	O ₂	competitive	catalase	$3 \cdot 10^{-9}$ – $1.5 \cdot 10^{-5}$ g/ml	Aizawa et al. (1979a)
	ferrocene	sandwich	GOD	$3 \cdot 10^{-11}$ – $3 \cdot 10^{-10}$ g/ml	Robinson et al. (1985)
	ferrocene	competitive	GOD	$1.3 \cdot 10^{-10}$ – $1.1 \cdot 10^{-8}$ g/ml	Robinson et al. (1986b)
Albumin	O ₂	competitive	GOD	$1 \cdot 10^{-8}$ mol/l	Mattiasson and Nilsson (1977)
IgG	O ₂	competitive	catalase	$6 \cdot 10^{-11}$ – $6 \cdot 10^{-9}$ mol/l	Aizawa et al. (1978)
	H ₂ Q/BQ	sandwich	GOD	$3 \cdot 10^{-13}$ mol/l	Gyss and Bourdillon (1987)
HBs	O ₂	sandwich	GOD	$1 \cdot 10^{-10}$ – $1 \cdot 10^{-7}$ g/ml	Boitieux et al. (1984)
	iodine	sandwich	HRP	$5 \cdot 10^{-10}$ g/ml	Boitieux et al. (1979)

AFP = α -fetoprotein; HCG = human chorionic gonadotrophine; IgG = immunoglobulin G; HBs = hepatitis B surface antigen; H₂Q/BQ = hydroquinone/benzoquinone

the immunological reaction was not allowed to reach equilibrium, but two minutes after sample injection the reaction chamber was rinsed and filled with the substrate of the marker enzyme (2 mmol/l H_2O_2 or 100 mmol/l glucose). The enzyme activity bound to the antigen-antibody complex could be detected at the oxygen probe within another two minutes. As in all competitive EIA the concentration of antigen to be determined was inversely proportional to the enzyme activity bound at the immobilized immunocomplex. The lower detection limit was 1 $\mu\text{mol/l}$ for insulin and 10 nmol/l for albumin, i.e., the sensitivity was significantly lower than that of conventional RIA. However, the sensor response was comparatively rapid and the sensor was easily handled.

Sensors for IgG (Aizawa et al., 1978), theophylline (Haga et al., 1984), and hepatitis B surface antigen (HB_S antigen) (Boitieux et al., 1984) are likewise based on the EIA technique with amperometric enzyme activity determination. The appropriate antibody membranes were regenerated after each measurement by decreasing the pH value of the solution. The membrane thus becomes suitable for a multitude of assays. In the sensors for IgG and theophylline the competition of catalase-labeled and unlabeled antigen leads to the generation of oxygen depending on the concentration of antigen. Oxygen is measured at the electrode. The sandwich method has been used in the sensor for HB_S -antigen. Gelatin-entrapped antibodies on the surface of an oxygen electrode react with HB_S -antigen from the serum sample. GOD-labeled antibody is added and bound, and the bound activity is measured via registration of the O_2 consumption after addition of glucose.

A relatively time-consuming immunoassay based on an enzyme immunoprobe has been developed by Aizawa et al. (1979a, 1980b). After a rather long incubation period of a membrane-immobilized antibody with the complementary antigen in a test tube the membrane is rinsed and attached to an oxygen probe. The bound enzyme activity is used as a measure of the antigen to be determined.

Recently, Boitieux et al. (1987) proposed a novel method for separating labeled immunocomplexes from free unlabeled antigen. A membrane capable of specifically binding β -galactosidase was fixed to an oxygen electrode. The antigen, IgG, competed with GOD-labeled IgG for β -galactosidase-labeled antibody. As soon as the complex was formed in the solution it was reversibly bound to the sensor surface. The GOD activity was inversely proportional to the concentration of IgG.

Similar sensors have been developed for the determination of human chorionic gonadotrophin (HCG) (Robinson et al., 1985, 1986b) and α -

fetoprotein (AFP) (Aizawa et al., 1980b). The respective membranes were only usable for one assay but the advantage of the methods lay in the fact that the analyzer was available for measurement during the incubation of a sample.

Robinson et al. (1985) improved the concept of enzyme immunoelectrodes by introducing magnetic carrier materials. In a sandwich assay for HCG, this permitted the separation of the GOD-labeled immunocomplex from the measuring solution by a magnetic working electrode. The activity of GOD was determined from the magnitude of the catalytic current of ferrocene oxidation which was thus directly proportional to the concentration of HCG in the range 0.25–2.5 U/l. The time required for one measuring cycle was 20 min. The assay has been simplified by direct binding of HCG antibody to a carbon electrode (Robinson et al., 1986b). In this case no separation was necessary and less antibody was needed. Up to 9 U/l of HCG could be determined with a measuring time for one assay of 20 min and good correlation with a radioimmunochemical method. Washing with 8 mol/l urea provided reusability of the sensor for about 40 measurements with no decrease of the reactivity of the antibody. The limit of the measuring range of 75 U/l was set by the number of antibody molecules covalently bound to the electrode surface. Enlarging or geometrically changing the electrode might therefore increase the amount of bound antibody and expand the dynamic range of the sensor.

In a similar approach, IgG concentrations in the femtomolar range have been detected by using a glassy carbon electrode (Gyss and Bourdillon, 1987). The bound GOD activity was measured after successive incubation of the sensor with sample antigen and GOD-labeled anti-IgG antibody. Between the measurements the surface of the electrode was cleaned electrochemically. Consequently, fresh antibody had to be adsorbed before each assay, a procedure that required about 2 h.

Durst and Blubaugh (1986) developed an immunoelectrode with double signal amplification based on the combination of complement-mediated liposome lysis with a chemically modified electrode (Fig. 123). The membranes of the liposomes, which included a dehydrogenase, were sensitized to antigen by entrapped antibody. The liposomes were co-immobilized with NAD^+ on the electrode. The antigen to be assayed was added to the measuring solution in the presence of complement and the dehydrogenase substrate. The formation of the immunological complex enables complement fixation leading to liposome lysis. Thus, a large number of enzyme molecules can freely diffuse into the solution where

they catalyze the reduction of NAD^+ . The NADH formed was electrochemically reoxidized to NAD^+ , the oxidation current being related to the analyte concentration.

Since the membranes used are only applicable to one or at most a few

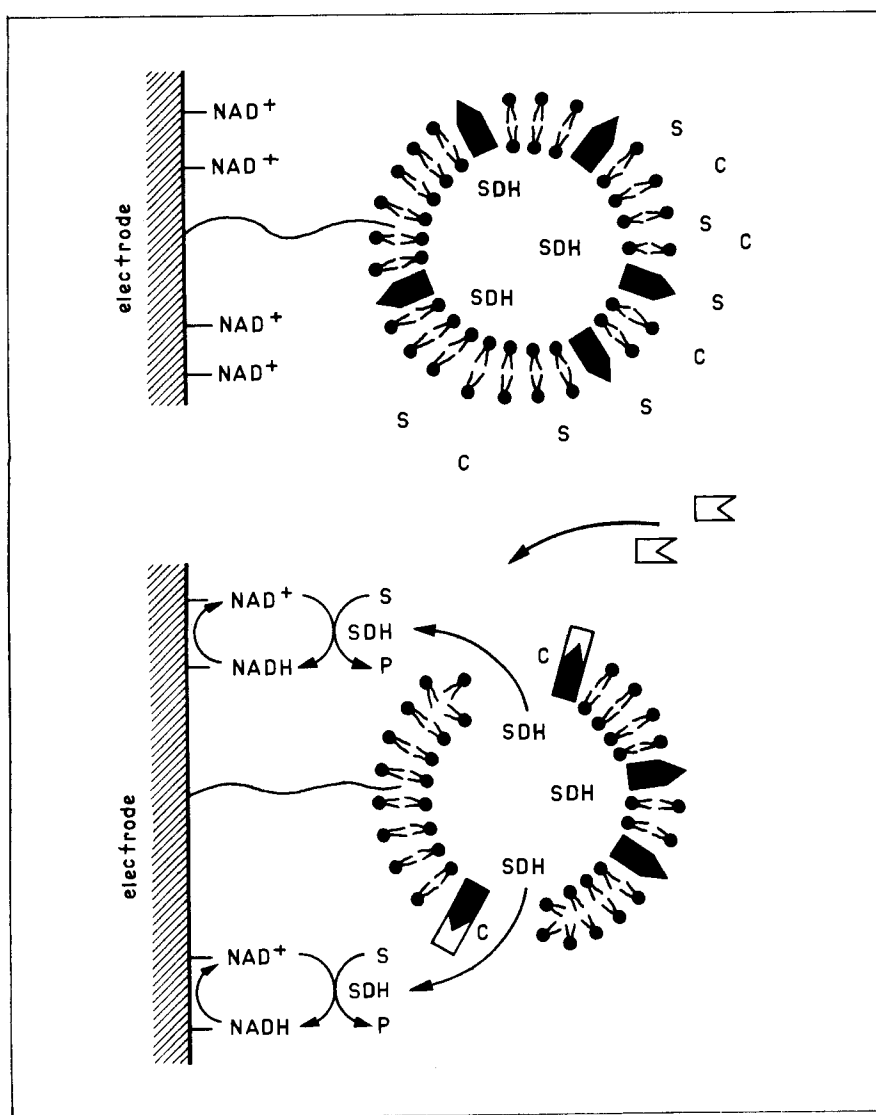


Fig. 123. Immunoelectrode with double signal amplification based on coimmobilized liposomes and NAD^+ . SDH = substrate dehydrogenase, C = complement, P_1 = lipid. (Redrawn from Durst and Blubaugh, 1986).

assays and the measuring cell is occupied during the formation of the complex, serial analyses, such as may be performed by using RIA, fluorescence immunoassays, or EIA, are not feasible with the immunoelectrodes described in this section. These sensors have thus not as yet been used in practical applications.

4.3.5 Reagentless Immunolectrodes

Biological macromolecules contain on their surface several positive and negative charges. Deposition of such molecules on electrodes therefore generates alterations of the electrochemical double layer. This results in potential shifts and changes of the dielectric constant in the vicinity of the electrode surface, the former being the basis of direct potentiometric analysis methods. Membrane electrodes with immobilized antibody, as well as electrodes directly covered with antibody, have been employed in the determination of macromolecules and haptens. Antigens have been coupled to the electrode for assaying antibodies. Attempts to use capacitance measurements to detect immunoreactants have also been made (Newman et al., 1986).

Ion selective membrane electrodes have been employed for the binding of antibodies against cortisol, digoxin, dinitrophenol (DNP), and serum albumin (Keating and Rechnitz, 1983, 1984; Solsky and Rechnitz, 1981). The appropriate antigen was conjugated with a benzo crown ether ionophore and the conjugate immobilized on a PVC membrane of 0.2 mm thickness, which was attached to a potassium ion-sensing probe (Fig. 124). The potential shift of the electrode was generated by changes of the ionophore resulting from antibody binding. For assaying anticortisol antibody, after formation of a stable base potential the sample was added and the potential change was registered after 3–9 min. The calibration curve between 3.5 and 165 ng/ml was not strictly linear. The antigen membrane electrode was regenerated by a brief rinse with citrate buffer, pH 4.0. Other proteins than anticortisol antibodies, such as gamma-globulins, did not cause nonspecific effects.

A similar procedure was used for antidigoxin antibody assay. Digoxin-benzo crown ether conjugates, rather than cortisol were entrapped in the PVC membrane of the K^+ sensitive electrode. The detection limit was in the range of a few g/ml. The sensor was regenerated by a brief (<60 s) immersion in glycine-HCl buffer, pH 2.8. The membrane was stable for 1–2 weeks under conditions of routine use.

A novel immunosensor type for low-molecular weight analytes has

been described by Bush and Rechnitz (1987). Antibody was immobilized directly on the membrane of a potentiometric electrode containing a hapten. The reversible competitive binding assay was carried out without use of labeled antigen in the sensor. In this manner, the antigen concentration could be continuously monitored. The feasibility of this principle has been demonstrated by assaying DNP. DNP was entrapped in the polymer layer of a K^+ -sensing electrode and covered with a

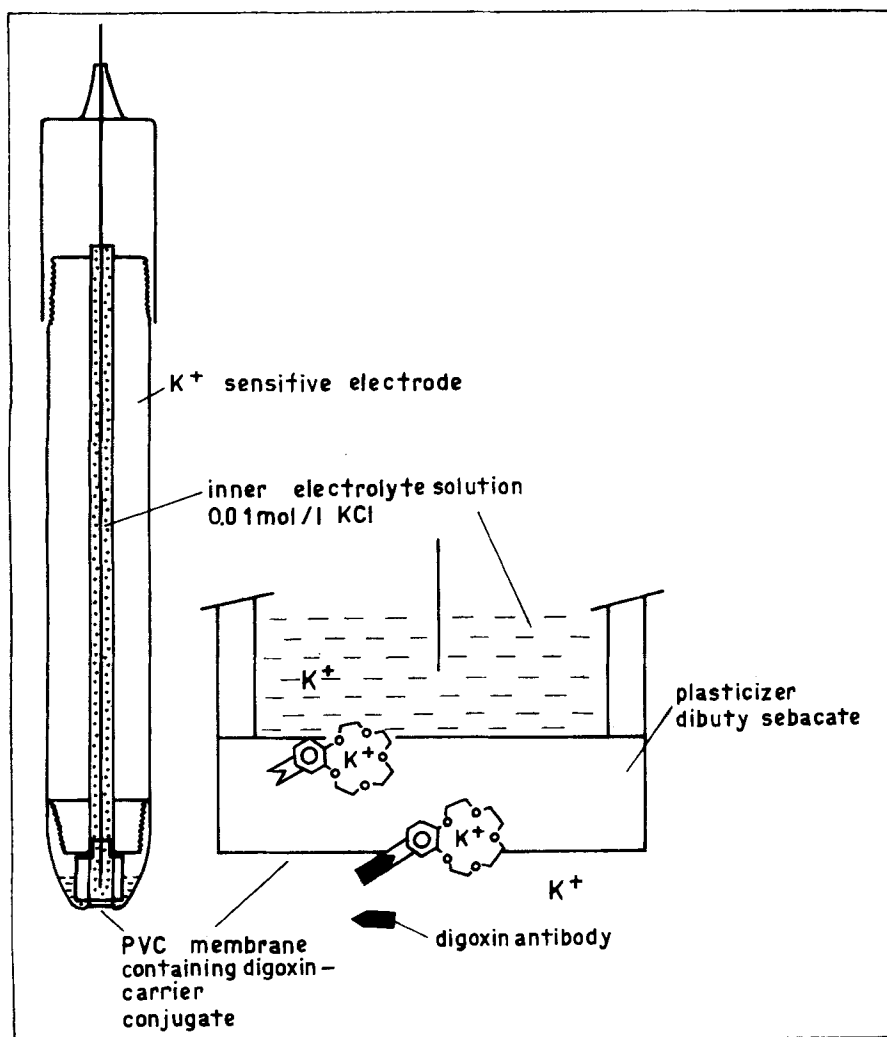


Fig. 124. Direct potentiometric digoxin antibody-sensing electrode. (Redrawn from Keating and Rechnitz, 1984).

collagen membrane incorporating monoclonal antibody against DNP. The sensor was inserted in a solution of constant potassium ion concentration. A stable base potential was formed due to the DNP-antibody binding occurring in the sensor membrane. Injection of DNP decreased the amount of antibody bound to the immobilized DNP by the fraction bound to added DNP. The resulting potential change reached a steady state after 15 min. As the immunological reaction is irreversible and occurs directly within the sensor, the analyte can be measured continuously. The sensor was useful for DNP analysis in the micromolar range and was stable for at least 17 days. Application to the assay of other low molecular weight haptens appears to be possible. Owing to the diffusion resistance of the membrane, the sensor is not usable for the measurement of high molecular weight substances.

Other, less specific potentiometric immunoprobes are based on the antigen-induced potential shift of chemically modified semiconductor electrodes (Fig. 125) (Yamamoto et al., 1983). The surface of a titanium dioxide electrode is covered by a BrCN-activated polymer membrane and inserted into an antibody-containing solution. The antibody binds covalently to the activated electrode surface. The antigen to be determined is added when the potential difference between the sensor and a reference electrode is stable. As a result of the immunological reaction a new

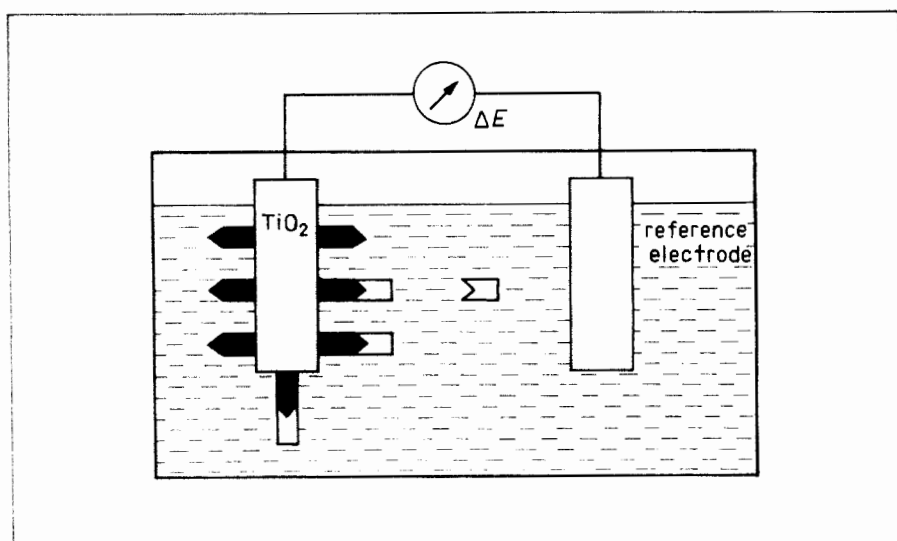


Fig. 125. Chemically modified semiconductor electrode with covalently bound antibody for antigen assay. (Redrawn from Yamamoto et al., 1983).

potential difference is formed after 20 min. The potential change is a measure of the antigen content of the sample. As a practical example, a monoclonal antibody selectively reacting with trinitrophenol group-containing substances, antitrinitrophenol IgA, has been fixed to the activated electrode. The sensor responded to trinitrophenol- γ -globulin in the concentration range 0.066–1 $\mu\text{mol/l}$ and was thus as sensitive as an enzyme immunoassay. Trinitrophenol-ovalbumin interfered.

Yamamoto et al. (1978) proposed the use of an antibody-coated titanium dioxide electrode to detect HCG in the urine of pregnant women. However, the measured potential of such sensors also depends on buffer composition, pH, and the ionic strength of the sample. Moreover, nonspecific adsorption of proteins at the transducer surface falsifies the measurement. This means that the accuracy of the analysis depends on the ratio of specific and nonspecific interactions.

Immuno Field Effect Transistors (ImmunoFETs)

In immunoFETs the gate of an ion selective field effect transistor is covered with an antibody-bearing membrane. Such sensors have been assembled for the assay of antialbumin antibody (Bergveld et al., 1987) and antisiphilis antibody (Janata and Huber, 1980). Owing to the poor selectivity, short lifetime, and mostly insufficient insulation of the semiconductor surface of the sensors this research is at present only conceptual. Furthermore, it is an intricate process to develop a completely insulating but immunoresponsive membrane thin enough to permit the detection of electric field variations exerted by the very small charge redistribution resulting from antibody–antigen binding. The manufacture of satisfactory membranes will require the use of new technologies that are as yet underdeveloped.

4.3.6 Piezoelectric Systems

Immunoassays using piezoelectric detectors coated with antigen or antibody had been devised as early as 1972 (Shons et al., 1972). The mass increase resulting from the immunological complex formation leads to a measurable change of the resonance frequency of the piezoelectric crystal. Effects of temperature and electronic noise can be eliminated by using a pair of crystals. Such sensors are only applicable in the dry state.

The above authors coated a piezoelectric crystal with BSA and measured the adsorption of BSA antiserum. The sensor responded to

anti-BSA antibody over a concentration range of three orders of magnitude. Similar results have been obtained by using the passive agglutination technique which, however, requires an incubation period of several hours, whereas the sensor method needs only a few minutes. On the other hand, the regeneration of the sensor requires extensive washing and drying.

Ngeh-Ngwainbi et al. (1986a) proposed an antibody-coated crystal for the measurement of parathion in the gas phase. Human IgG has been determined in concentrations between 10^{-11} and 10^{-7} mol/l by using a quartz upon which protein A was immobilized by means of γ -amino-propyl triethoxysilane (Muramatsu et al., 1987b). This immobilization method has also been used to construct a sensor for the determination of microorganisms (Muramatsu et al., 1986). The sensor surface was anodically oxidized and coated with palladium before immobilization of anti-*Candida* antibody. The affinity binding of pathogenic *Candida albicans* resulted in a decrease of the resonance frequency related to the amount of cells between 10^6 and $5 \cdot 10^8$ in the range 0.5–1.4 kHz. Since dry crystals had to be used, the measuring time was as long as 1 h. This drawback has been avoided in a system for the detection of nanogram-amounts of human IgG and influenza type A-virus by measuring in a solution versus an uncoated reference crystal (Roederer and Bastiaans, 1983). The sensor responded to the analyte within 20 s. It could be regenerated with a solution of high ionic strength, so that the antibody-coated crystal surface was available for multiple assays. Unspecific adsorption of sample constituents at the indicator and reference crystals significantly decreased the sensitivity. The sensor was only applicable to the assay of high molecular weight substances.

4.3.7 Optical Immunosensors

Antigen–antibody reactions at surfaces are accompanied by changes in certain optical properties which forms the basis of optoelectronic immunosensors. The thickness of a monolayer of antigen–antibody complex amounts to about 20–40 nm. The change of the layer thickness, refractive index, light absorption in the layer, reflective behavior of the incident light, and light scattering are used to generate measuring signals related to antigen concentration. Sophisticated methods such as ellipsometry, internal reflection spectroscopy, fluorescence spectroscopy, and surface plasmon resonance spectroscopy have been employed for the registration of these signals (Place et al., 1985).

Ellipsometry is a widely used method for the investigation of thin layers on reflecting surfaces. Owing to its large refractive index, silicon is well suited as a supporting material, its surface frequently being metallized by evaporation. When a molecular layer of some nanometers' thickness is adsorbed, the reflection of polarized monochromatic light at the surface changes due to attenuation and phase shifting. Reflectometers are being used for kinetic studies of binding reactions, e.g., of antibodies to surfaces or to antigens. As shown with IgG as model analyte, adsorption of surface protein concentrations as low as $0.1 \mu\text{g}/\text{cm}$ can be detected (Welin et al., 1984).

The method has been used for the determination of cholera toxin (Stenberg and Nygren, 1982), anti-human serum albumin (Arwin and Lundström, 1985), BSA (Elwing and Stenberg, 1981), fibrinogen (Cuy-pers et al., 1978), *Leishmania donovani* (Mathot et al., 1967), and various microbial and viral antigens such as polysaccharides of pneumococci (Rothen, 1947; Rothen and Mathot, 1971). The appropriate complementary biomolecule was adsorbed onto the hydrophobic silicon or metal (oxide) surface. The immunological reaction can be accelerated by combining ellipsometry with immunoelectroadsorption methods. Application of a current of $300 \mu\text{A}$ during adsorption and immunological reaction has been shown to increase the sensitivity by up to six orders of magnitude, thus enabling the detection of as little as $0.2 \text{ ng}/\text{ml}$ of human growth hormone (Rothen et al., 1969). The reproducibility of such methods is affected by the instability of the metal oxide surface layer. In order to avoid nonspecific binding events, the gel diffusion method has been used in ellipsometric measurements (Elwing and Stenberg, 1981).

Seifert et al. (1986) prepared a planar $\text{SiO}_2\text{-TiO}_2$ wave guide with a surface relief by using an embossing technique. The waveguide reacts sensitively to the surface adsorption of proteins. IgG has been adsorbed and the binding of anti-IgG was registered with a difference refractometer.

The increase in the intensity of light scattered from a glass surface coated with an indium film and antibody has been monitored to measure a rheuma factor (Giaever et al., 1984). The light scattering was proportional to the antigen concentration.

Another technique that is important for optical immunosensor development is internal or attenuated total reflection spectroscopy (Fig. 126). A waveguide (slide or fiber) having a high refractive index is coated with a layer of immobilized antibody. The incident light

interacts with the layer resulting in a change of the critical angle for total reflectance. The incident beam passes through the waveguide by multiple internal reflections and interacts several times with the sample film. At each reflection the evanescent component of the completely internally reflected light wave of the order of a fraction of the wavelength penetrates the coating of the waveguide. Thus the absorption of the evanescent component is mainly due to constituents that are very close to the surface. The method has been employed in a homogeneous immunoassay for human IgG (Sutherland et al., 1984). With rapid kinetic measurement the detection limit was 20 ng/ml. Continuing this development, the authors constructed an in-line sensor for IgG that was largely independent of environmental disturbances (Sutherland et al., 1987). The sensor was based on the competition of IgG immobilized on the waveguide with the analyte for anti-IgG-fluorescein isothiocyanate.

Total internal reflection fluorescence spectroscopy has been used to assay the fluorescence of tryptophan in proteins or of fluorescence markers. Morphine has been determined in this manner with a detection limit of $0.2 \mu\text{mol/l}$ on a quartz support bearing immobilized fluorescein-labeled antihapten (Kronick and Little, 1973).

Various detection systems have been constructed employing surface plasmon resonance spectroscopy. A surface plasmon is a collective motion of electrons in the surface of a metal conductor, excited by the impact of light of an appropriate wavelength at a particular angle. For a given wavelength of light a surface plasmon effect is observed as a sharp minimum in the light reflectance when the angle of incidence is varied. The critical angle is very sensitive to the binding of analytes to the metal surface. The particular optical

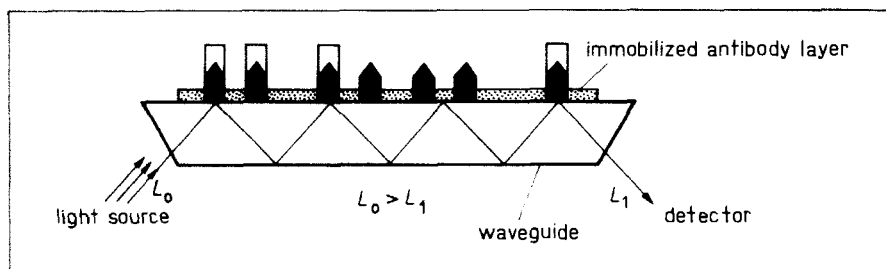


Fig. 126. Internal reflection spectroscopy for antigen determination.

conditions required for generation and use of surface plasmons for immunosensing applications have been achieved using metallized diffraction gratings and using the metallized surface of glass prisms. By reason of its chemical stability in aqueous buffer, gold is the preferred metal. The minimum detectable layer thickness change is 1 nm (Nylander et al., 1982). Model immunoprobes have been designed for the assay of IgG in concentrations around 2 $\mu\text{g/ml}$ within a few seconds (Liedberg et al., 1983), and for assay of anti-HSA (Flanagan and Pantell, 1984; Kooyman et al., 1987).

4.4 BIOSENSORS USING INTACT BIOLOGICAL RECEPTORS

The interfacing of biologically intact sensory structures to electrodes leads to an area of bioelectrochemistry that lies on the borderline between neuroelectrophysiology and biosensor technology. In the coupling of chemoreceptors as molecular recognition elements with electrodes — which are then named receptrodes — two directions may be distinguished, one dealing with isolated receptors and the other with complete chemoreceptor structures. These levels of integration are comparable to enzyme electrodes on the one hand and sensors using higher integrated biocatalytic systems on the other. In contrast to enzymes, however, only a few receptors have as yet been isolated in very small amounts.

Isolated nicotonic acetylcholine as well as plant receptors have been employed in receptrodes for the assay of acetylcholine and its antagonists, and auxin and toxin, respectively (Rechnitz, 1987; Thompson et al., 1986).

An ion sensitive field effect transistor has also been used as a transducer (Gotoh et al., 1987). The acetylcholine receptor was fixed at the gate of the transistor by using a poly(vinyl butyrate) membrane. Binding of acetylcholine in the concentration range 0.1–10 $\mu\text{mol/l}$ caused potential changes that were detectable with the transistor. By embedding the receptor in a lipid membrane a threefold sensitivity enhancement was obtained due to the generation of an acetylcholine-dependent sodium ion flux through the receptor channel. Owing to their inherent instability, short lifetime, and poor reproducibility such sensors are not practically useful at present.

Eldefrawi et al. (1988) devised a sensor composed of the acetylcholine

receptor from the electric organ of the Torpedo fish and a planar interdigitated capacitive sensor. The chromium fingers of the sensor had a width and a separation of approximately $50\text{ }\mu\text{m}$. The capacitive surface was passivated by deposition of a glass layer of $0.25\text{ }\mu\text{m}$ thickness. A lipid layer was deposited on the capacitive surface by dip coating followed by adsorption of the receptor from the solution. The capacitance of the sensor increased upon addition of acetyl choline in the concentration range $1\text{--}100\text{ }\mu\text{mol/l}$. The competitive antagonist d-tubocurare and the noncompetitive antagonist amantadine inhibited the response to acetylcholine.

An alternative to the use of isolated receptors which provides higher sensor stability is the use of intact sensory structures of living organisms. The stimulation of certain receptors of such sensory structures generates measurable action potentials in neurons. Olfactory organs in the antennules of the blue crab, *Calinectes sadipus*, contain chemoreceptors that respond to amino acids. The chemical information is neurally coded and can therefore be recorded electrophysiologically. The antennules of such a crab have been employed as active sensing elements in a receptrode for amino acid determination (Belli and Rechnitz,

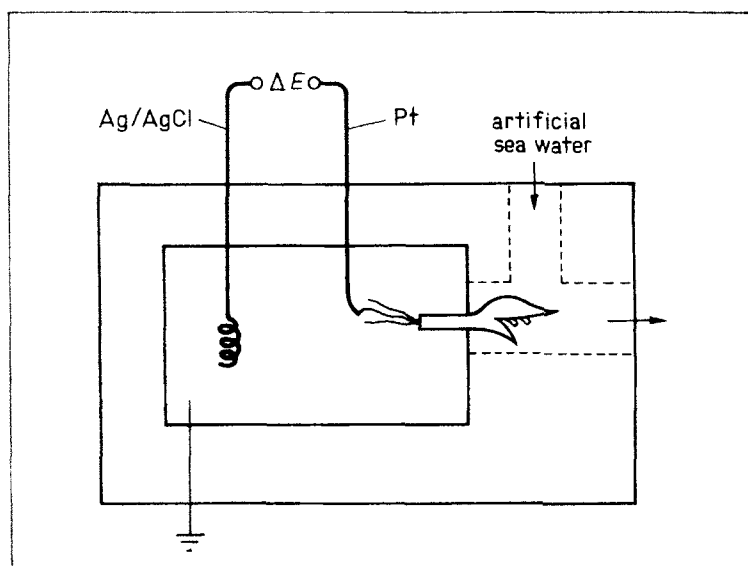


Fig. 127. Receptrode containing intact blue crab olfactory organ. (Redrawn from Belli and Rechnitz, 1986).

1986). A platinum probe was coupled to a single nerve fiber or to a nerve fiber bundle of the antennule (Fig. 127). Stimulation of the sensor by L-glutamate created nerve impulses having amplitudes between 10 and 1000 μV within 5–10 seconds. Quantitative data of the multiunit response were gained by integrating the signal and summing the area under the response curves. Single unit recordings could be analyzed by counting the discrete pulses. The sensor response was linearly dependent on the concentration of L-glutamate in the range of 0.01 to 1 mmol/l. The lowest detectable concentration was 1 $\mu\text{mol/l}$.

Among other effects the interaction of hormones with receptors in intact biological membranes causes activation of adenylate cyclase and permeability changes. This behavior has been utilized in a sensor for human chorionic gonadotrophin comprising toad bladder and a Na^+ -sensing glass electrode (Updike and Treichel, 1979). When the hormone to be measured was bound at the antiluminal side to the receptor, the formation of cAMP was stimulated. The water permeability of the membrane and the antiluminally directed Na^+ flux therefore increased. The resulting potential change could be further increased by adding mannitol to the internal solution between the mucous membrane and the electrode, thus increasing the osmotic pressure. The gradient between the internal and the external solution resulted in a water flux which additionally diluted the internal sodium ion solution and thus decreased the potential. With a linear calibration graph for chorionic gonadotrophin between 5 and 30 mU/ml the upper limit of detection was 40–50 mU/ml.

Neutrophil leucocytes respond to various stimulants by the production of extensive amounts of superoxide anion, the so-called respiratory burst. A sensor for neutrophils based on the stimulation by IgG adsorbed on a pyrolytic graphite electrode has been described by Green et al. (1984). The liberation of superoxide anion as detected within 3 min at the sensor was related to the number of neutrophil leucocytes. The absence of an oxidation current in the presence of superoxide dismutase proved the formation of superoxide anion.

A promising approach has been proposed by Sugawara et al. (1987), who used the Langmuir-Blodgett technique to transfer lipid membranes mimicking ion channels to a carbon electrode surface. Stimulation of the channels by Ca^{2+} makes the layer permeable to ferrocyanide, the oxidation current of which is related to the concentration of Ca^{2+} in the measuring solution. Inclusion of biological receptors in artificial lipid membranes, which is feasible by using the Langmuir-Blodgett method

(Roberts, 1983; Wingard, 1987), might greatly facilitate the development of sensitive and selective receptor-based sensors for the respective ligands.

Chapter 5

Application of Biosensors

5.1 GENERAL ASPECTS

Between US \$12 and 15 billion per year is spent worldwide for analytical purposes; the portion used for enzymes amounts to about \$50 million. Enzymes are being employed in clinical chemistry, the food and cosmetic industries, and biotechnology for the routine analysis of about 80 different substances, mainly low-molecular weight metabolites but also effectors, inhibitors, and the activity of enzymes themselves.

A broad spectrum of immunoassays for low-molecular weight haptens, macromolecules, and microorganisms have been made available in recent years through the enormous progress in immunological research, especially in the preparation of monoclonal antibodies. About one billion immunoassays are sold per year.

The development of immobilization methods has given an impetus to the routine use of enzymes and antibodies in analytical chemistry. The main advantages of these immobilized reagents are their reusability, simple and safe handling, and the possibility of spatially restricting the bioanalytical reaction so as to achieve a significant simplification of the analytical apparatus. A breakthrough of biosensors can be expected in areas where the investments for their development rapidly amortize and high economic benefits are guaranteed. One of the most promising fields of biosensor application is biotechnology.

Based on the sensor technologies developed for these purposes the application will expand enormously to other areas of chemical industry. According to a prognosis by Tschannen et al. (1987), in 1990 the biosensor market in Western Europe will rise to US \$440 million. Worldwide, a potential market for 500 million glucose sensors is anticipated.

A relevant aspect in biosensor research is the simplification of operation, the more so as test strips are at present still superior in this respect.

The savings of reagents provided by reusable sensors should not be exceeded by the expenses necessary for sensor maintenance. The question is whether reusability and simple handling can be combined in *reusable test strips* or *disposable biosensors*. The first concept leads to optoelectronic biosensors; the second has gained increasing attention too, inasmuch as disposable chemical sensors can be manufactured by using mass production technology. At present, cheap disposable biosensors based on thin film electrodes appear to be more promising than enzyme field effect transistors. As compared with test strips, disposable biosensors are advantageous in that only an undefined amount of sample has to be applied, no exact measuring regime is required, and the sample does not have to be removed from the sensor. However, since the user will scarcely be able to calibrate the sensors, they have to be virtually identical within one batch.

The integration of electronic signal processing and display into disposable sensors appears to be difficult. Hybrid systems comprising a disposable sensor and a separate, portable device are likely to prevail.

Whereas in traditional enzymatic analysis spectrophotometric methods dominate, test strips and biospecific electrodes are at the leading edge in the analytical application of immobilized enzymes. This may be expected to continue at least until the mid-90s.

5.2 BIOSENSORS FOR CLINICAL CHEMISTRY

Most clinical laboratory analyses concern metabolites in blood and urine in the micro- and millimolar concentration range. A better understanding of several diseases requires the measurement of steroids, drugs and their metabolites, hormones, and protein factors. Since they lie in the range of 10^{-11} to 10^{-9} mol/l, the concentration of these substances can at present only be determined by using immunoassays. Stat determination and continuous in vivo monitoring of these substances are particularly important in intensive care medicine, surgery, and life-threatening situations.

5.2.1 Test Strips and Optoelectronic Sensors

Nowadays, test strips for the determination of about ten low-molecular weight substances (metabolites, drugs and electrolytes) and eight enzymes are available on the market (Libeer, 1985), one strip usually costing more than US \$1. Pocket photometers and computerized photometric and potentiometric devices are being offered as readout instru-

ments. However, neither visual nor pocket-photometric evaluation provide the analytical quality achieved in automated enzymatic analysis. On the other hand, application of enzyme test strips in semi-automatic analyzers appears to be economically disadvantageous. Therefore, test strips are mainly employed in home monitoring and screening in the doctor's consulting room and in small clinical laboratories.

Optoelectronic biosensors based on immobilized dyes have been developed for the determination of glucose, urea, penicillin, and human serum albumin (Lowe et al., 1983). Other promising approaches use immobilized luciferase or horseradish peroxidase to assay ATP or NADH or, when coupled with oxidases, to measure uric acid or cholesterol. These principles have not yet been generally accepted for use in routine analysis. Most probably, the first commercial optical biosensors will be those for immunological assays.

5.2.2 Thermistors

Although thermistor devices involving the use of immobilized enzymes or antibodies for a number of clinically relevant substances have been described (Table 22), their practical use is at present limited to a few research laboratories. Thermometric enzyme linked immunosorbent assays are being routinely employed in monitoring the production of monoclonal antibodies. A broad application is restricted by the low sample throughput and the high equipment costs.

5.2.3 Enzyme Electrodes

Between 15 and 20 analyzers based on enzyme electrodes are on the market worldwide. They are one-parameter instruments for the measurement of glucose, galactose, uric acid, choline, ethanol, lysine, lactate, pesticides, sucrose, lactose, and the activity of α -amylase (Table 23). They provide for a negligible enzyme consumption of less than 1 μg per sample.

The Glukometer GKM 01 (Zentrum für Wissenschaftlichen Gerätebau, Academy of Sciences of the GDR) was the first commercial enzyme electrode-based glucose analyzer developed in Europe. It was introduced in 1981. At present 300 of these instruments are being employed for blood glucose determination in the medical sector. Furthermore, the Glukometer is being adapted to the quantification of uric acid, lactate, and the activity of acetylcholine esterase. Its applicability to the assay of seven more analytes is being tested (Table 24).

TABLE 22

Enzyme Thermistors

Substance	Immobilized biocatalyst	Measuring range (mmol/l) or detection limit
<i>Clinical chemistry</i>		
Ascorbic acid	ascorbate oxidase	0.05–0.6
ATP	apyrase or hexokinase	1–8
Cholesterol	cholesterol oxidase	0.03–0.15
Cholesterol ester	cholesterol esterase + cholesterol oxidase	0.03–0.15
Creatinine	creatinine iminohydrolase	0.01–10
Glucose	glucose oxidase + catalase	0.002–0.8
Glucose	hexokinase	0.5–25
Lactate	lactate monooxygenase	0.01–1
Oxalic acid	oxalate oxidase	0.005–0.5
Oxalic acid	oxalate decarboxylase	0.1–3
Triglycerides	lipoprotein lipase	0.1–5
Urea	urease	0.01–500
Uric acid	uricase	0.5–4
<i>Immunological analysis (TELISA)</i>		
Albumin (antigen)	immobilized antibody + enzyme-labeled antigen	10^{-10}
Gentamicin (antigen)	immobilized antibody + enzyme-labeled antigen	0.1 µg/ml
Insulin (antigen)	immobilized antibody + enzyme-labeled antigen	0.1–1 U/ml

The Lipid Analyzer ICA-LG 400 from the Japanese company Toyo Jozo is capable of measuring a whole group of analytes, namely cholesterol, triglycerides, and phospholipids by using enzyme electrodes. Serum samples have to be preincubated with the appropriate hydrolases, i.e. cholesterol esterase, lipoprotein lipase, and phospholipase D. The measurement is performed by using enzyme electrodes involving cholesterol oxidase, glycerokinase (EC 2.7.1.30), glycerophosphate oxidase (EC 11.1.3.-), and choline oxidase immobilized in front of an oxygen probe. Using a sample volume of 30 µl a measuring frequency of 40/h is obtained. Although the prospects for this method appear exciting, the analyzer has not yet reached the market.

TABLE 23

Enzyme Electrode-Based Analyzers

Company, country	Model	Analyte	Linear range (mmol/l)	Sample frequency (h ⁻¹)	Serial CV (%)	Stability
Yellow Springs Instrument Co., USA	23A	glucose	1-45	40	<2	300 samples
	23L	lactate	0-15	40		
	27	ethanol	0-60	20	<2	
		lactose	0-55	20	2	
		galactose	0-55	20	2	
		sucrose	0-55	20	2	
Zentrum für Wissenschaftlichen Gerätebau, GDR	Glukometer	glucose	0.5-50	60-90	1.5	>1000 samples
	GKM	uric acid	0.1-1.2	40	2	10 days
Fuji Electric, Japan	GLUCO 20	glucose	0-27	80-90	1.7	500 samples
		α -amylase		30	4-5	
	UA-300A	uric acid		50-60	3	
Daiichi, Japan	AutoSTAT	glucose	1-40	60-120	3	
	GA-1120					
Radelkis, Hungary	OP-GL-7110S	glucose	1.7-20	40	5-10	240 days
USSR	ExAn	glucose	2.5-30	20	>3	
La Roche, Switzerland	LA 640	lactate	0.5-12	20-30	<5	40 days
Omron Tateisi, Japan	HER-100	lactate	0-8.3		<5	>10 days
Seres, France	Enzymat	glucose	0.3-22	60		
		choline	1.0-29	60		
		L-lysine	0.1-2	60		
		D-lactate	0.5-20	60		
Tacussel, France	Glucose-processeur	glucose	0.05-5	90	<2	>2000 samples
Prüfgeräte-Werk Medingen, GDR (Eppendorf, FRG)	ADM 300	glucose	1-100	80	<2	>2000 samples
	ECA 20 (ESAT 6660)	glucose	0.6-60	120-130	<1.5	10 days
		lactate	1-30	120	<2	14 days
		uric acid	0.1-1.2	80	<2	10 days

TABLE 24

Application of the Glukometer GKM

Analyte	Enzyme	Measuring range		Sample frequency (h ⁻¹)	Serial CV (%)	Stability (days)
		(mmol/l)	(U/l)			
Lactate	LOD	1-40		60	1	14
Pyruvate (+lactate)	LDH+LMO	0-7		20	2	55
Glucose	GOD	0.5-50		60-90	1.5	10
Urea	urease	0.8-50		40	1	15
Uric acid	uricase	0.1-1.2		40	2	10
Lactose	GOD+ β -gal	1-50		100	1.5	20
Maltose	GOD+GA	1-50		60	2	14
Sucrose	GOD+MR+ IN	1-44		40	1.5	5
Glutamate	GLOD	0.04-40		40	1.5	10
Phosphate	GOD+AcP	2-24		12	2	28
Lactate dehydrogenase	LMO		60-1200	15	2	55
Pyruvate kinase	LDH+LMO		60-840	15	3	55
Creatine kinase	PK+LDH+ LMO		60-1050	10	3	14
Acetylcholinesterase			200- 24000	40	2	21

LMO = lactate monooxygenase, LDH = lactate dehydrogenase, PK = pyruvate kinase, GOD = glucose oxidase, GLOD = glutamate oxidase, β -gal = β -galactosidase, GA = glucoamylase

5.2.3.1 Glucose

The prevalence of diabetes in industrialized countries amounts to approximately 4%. Therefore the selective determination of blood glucose is of utmost importance for the screening and treatment of diabetes. The normal concentration of glucose in blood serum ranges between 4.2 and 5.2 mmol/l.

Glucose analyzers based on enzyme electrodes are being marketed in the United States, Japan, France, the USSR, and Germany (see Table 23).

Similar to other analyzers the Glukometer GKM (Fig. 128) is particularly well suited to the analysis of single samples and small sample series. The measuring and sample solutions are injected by using pipettes. The preparation of the enzyme electrode requires about 3 min; the

sensor is stable for at least one week. A fresh enzyme electrode is ready for use after two or three calibrations. Further calibration is necessary every 20 samples.

The use of untreated whole blood as sample material would be a considerable advantage over that of plasma or prediluted samples. Studies using the Glukometer have shown that with undiluted blood the measured values are 19.8% lower than those obtained with 1:10-diluted blood (Scheller et al., 1986b). Obviously, without dilution the glucose content of erythrocytes is not completely accounted for (Fig. 129). Hanke et al. (1987) therefore suggested diluting the blood samples at a ratio of 1:10 to 1:50. Dilution of 500 μ l blood 1:10 with isotonic phosphate buffer containing 2 g/l dextran provides good analytical quality under conditions of routine use (Wolf and Zschesche, 1986). The average percentage



Fig. 128. Manual glucose analyzer Glukometer GKM (Zentrum für Wissenschaftlichen Gerätebau, Academy of Sciences of the GDR) consisting of a thermostat furnished with measuring cell and enzyme electrode (left), sample dispenser (center), and electronics (adapted to glucose, uric acid, and lactate measurement, right).

serial imprecision was 1.7%, the day-to-day imprecision being about 3%. For hospital samples excellent agreement was obtained with the o-toluidine method.

In contrast, the glucose analyzers of Yellow Springs Instrument Co. (YSI), Fuji Electric, and Daiichi measure only the true glucose concentrations in plasma and serum samples. Upon direct injection of whole blood into the Fuji instrument the measured values are too low by 13% (Niwa et al., 1981). This trend is confirmed by the correlation equation obtained with the AutoSTAT instrument of Daiichi as applied to whole blood samples:

$$y = (0.793x + 0.47)\text{mmol/l}$$

To correct for these systematic deviations, for use of the YSI analyzer a table is required that takes account of the hematocrit (Mason, 1987).

Based on investigations by Bertermann et al. (1981), a complementary glucose module for the automatic flow stream analyzer ADM 300

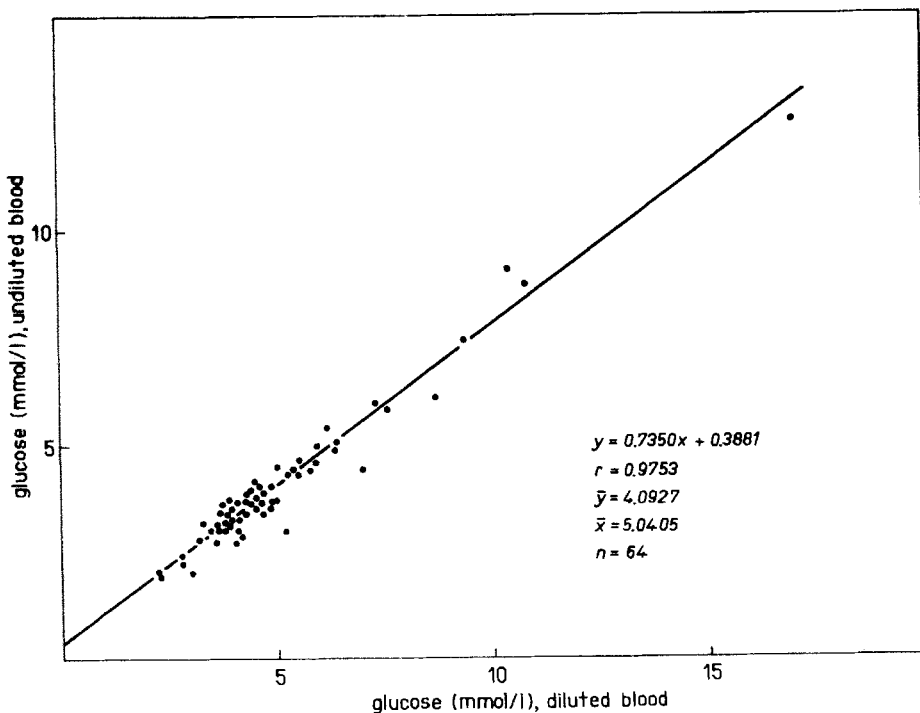


Fig. 129. Correlation of glucose measurement with the Glukometer using undiluted and 1:10 diluted blood samples.

(VEB Prüfgeräte-Werk Medingen, GDR) has been developed and clinically tested (Fig. 130). The instrument permits the determination of 80 samples per hour with a CV between 1.0 and 1.5% and is thus well suited to the processing of large sample series in centralized laboratories. The required stability of glucose in the sample is achieved by dilution with a hypotonic buffer of the following composition:

dextran M:	2 g/l,
EDTA:	977 $\mu\text{mol/l}$,
disodium hydrogen phosphate:	9.77 mmol/l,
potassium chloride:	19.5 mmol/l,
potassium dihydrogen phosphate:	2.93 mmol/l,
sodium azide:	0.977 mmol/l.

In this solution the sample hemolyzes immediately. The blood glucose concentration remains stable for 24 h. Glucose assay using this buffer

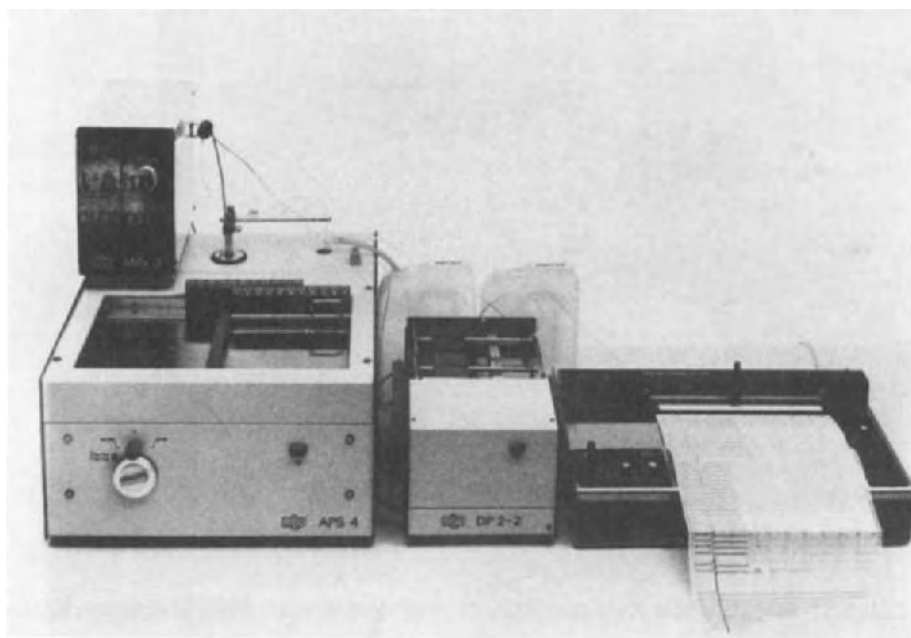


Fig. 130. Complementary module for glucose for the automatic flow stream analyzer ADM 300 (VEB Prüfgeräte-Werk Medingen, GDR) consisting of the automatic sampler APS 4, electronic amplifier AMV 3 with flow-through cell (left), peristaltic pump, and recorder.

gives values which agree well with those determined by standardized methods.

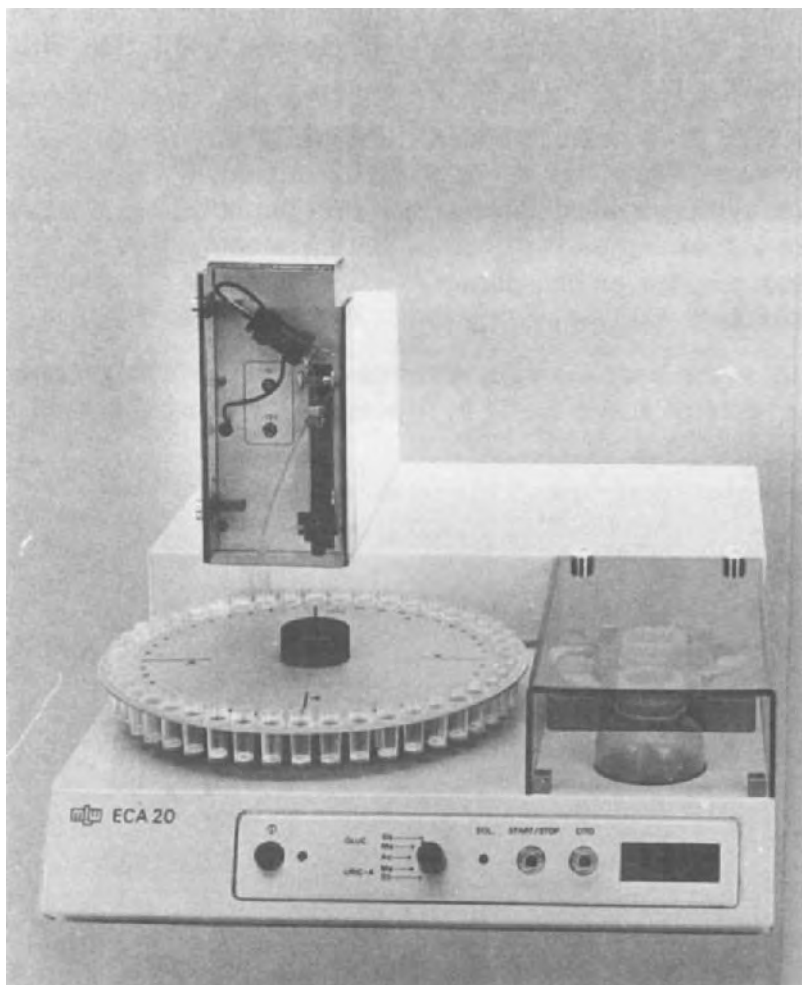


Fig. 131. Enzyme-Chemical Analyzer ECA 20 (VEB Prüfgeräte-Werk Medingen, GDR).

Based on the experiences gained during five years of routine use of the Glukometer, the Central Institute of Molecular Biology of the Academy of Sciences of the GDR and Prüfgeräte-Werk Medingen developed the microcomputer-based Enzyme-Chemical Analyzer ECA 20 shown in Fig. 131. This instrument is suited for glucose determination in the

concentration range 0.6–60 mmol/l with a day-to-day CV below 3% and an excellent correlation with the highly specific glucose dehydrogenase method (Fig. 132):

$$y = [(1.003 \pm 0.006)x - (0.015 \pm 0.045)] \text{ mmol/l};$$
$$r = 0.996 \text{ } (n = 196).$$

120 samples can be processed per hour; a stat-value can be obtained within 60 s. Calibration is performed automatically and only 5–20 μl of blood is required for a double determination. The glucose oxidase membrane used is stable for at least 2000 measurements. These parameters demonstrate the superiority of the device over other enzyme electrode-based analyzers. A modified variant of this instrument named ESAT 6660 is marketed by Eppendorf (FRG) (Fig. 133).

Hydrogen peroxide detection in enzyme electrodes for urine glucose assay is subject to severe interference by reducing substances, the more

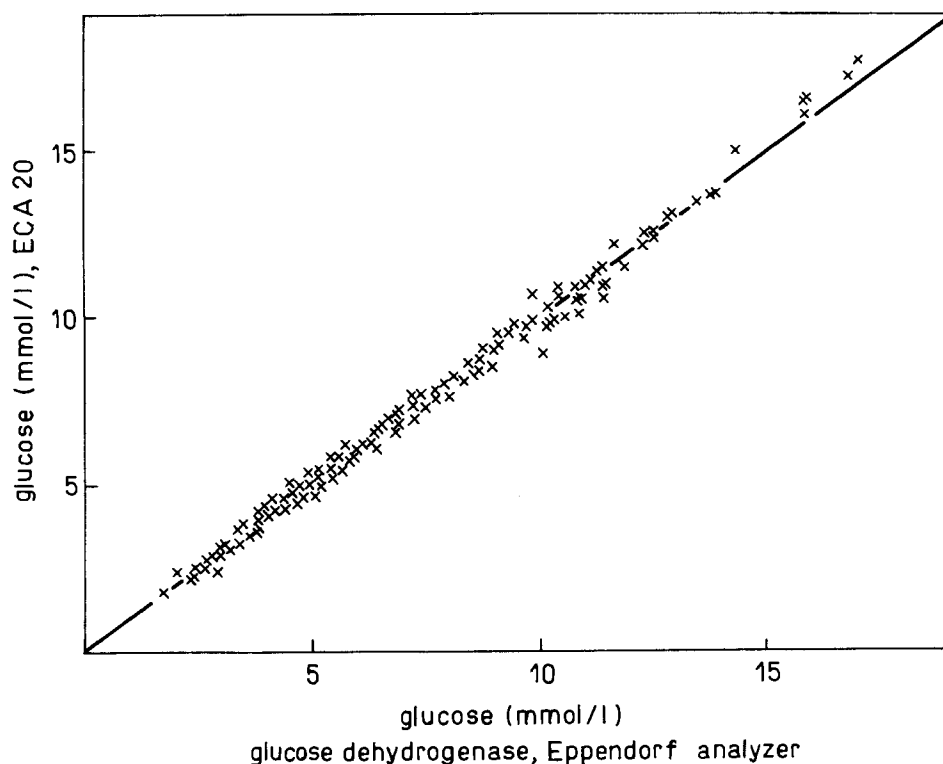


Fig. 132. Correlation of glucose measurement with the ECA 20 and the glucose dehydrogenase method using 1:50 diluted blood samples.

so as the normal glucose concentration of glucose in urine is only 0.2 mmol/l. The measuring values therefore agree with those of the hexokinase method only above 5 mmol/l (Jänchen et al., 1980). Such interferences can be eliminated by using a combined sensor method involving a cellulose nitrate-modified GOD membrane and the above mentioned hypotonic buffer, additionally containing 2 mmol/l ferrocyanide (Hanke, 1989). The buffer solution is capable of oxidizing disturbing substances to electrochemically inert products, thus leading to good agreement of the glucose values with those found by using the hexokinase method. Another favorable consequence of using this set-up is the opportunity to measure glucose in both urine and blood without the need to change any part of the instrument.

Recently, the first second-generation commercial glucose sensor has been introduced by Britain's Genetics International (McCann, 1987). The sensor is based on a ferrocene-modified GOD electrode strip (see Section 3.1.1.3). For glucose determination a drop of blood is transferred to the strip which is then inserted into a pen-sized readout instrument. The response time is only 30 s and thus much more rapid than that of



Fig. 133. Analyzer ESAT 6660 (Eppendorf, FRG).

test strips. Venous as well as capillary blood may be used as sample material. The CV is 3.9% for the normal concentration range. Significantly lower precision has been found in the hypoglycemic range. The following correlation to a (nonspecified) method was obtained:

$$y = (1.04x + 0.9) \text{ mmol/l}; r = 0.985.$$

The simple handling makes the sensor very well suited to use in the physician's consulting room and for home health care. The cost of US \$1 per measurement is rather high, however.

5.2.3.2 Urea

The concentration of urea in blood (blood urea nitrogen, BUN) is an important parameter in clinical chemistry in the assessment of kidney failure. The normal levels of urea in serum are 3.6–8.9 mmol/l.

Hamann (1987) employed a potentiometric urea electrode in an enzyme difference analyzer for urea determination in serum. The difference between the potential changes of a urease-covered and a bare pH glass electrode is evaluated 30 s after sample injection. This fixed-time regime provides a measuring frequency of 20–25/h; the linear range for 1:120 diluted samples is 1–20 mmol/l. These results are better than those of common potentiometric enzyme sensors.

For serum measurement the sensor system is calibrated with urea dissolved in 0.0185 mol/l Tris-HCl buffer, pH 7.0 ($\beta = 8 \text{ mmol/l}$). In this manner, disturbances caused by individually different sample pH values and variations in the buffer capacity are kept within the noise limits of the measuring system. Comparison with the Berthelot method resulted in the following equation:

$$y = (1.025x - 0.042) \text{ mmol/l}; r = 0.998 (n = 23).$$

The CV for 20 successive determinations in serum with a urea concentration of 6.5 mmol/l was 2.1%. The urease sensor had a useful lifetime of 28 days when stored at room temperature between measurements.

Petersson (1988b) developed an efficient urea analyzer for undiluted blood samples by using a urease-covered ammonium ion selective electrode in an FIA system. Forty samples per hour could be determined with a useful measuring range up to 40 mmol/l and a serial CV of 1%. The sensor was stable for 25 days. The correlation coefficient with a routinely used method was 0.99. Variations in the hematocrit level had only a small effect on the measurement.

An amperometric urea sensor based on the pH dependence of the anodic oxidation of hydrazine (Kirstein, 1987) has been utilized in the Glukometer GKM 02 for hemodialysis monitoring. For urea concentration in dialyzate the following correlation was obtained with the Berthelot method:

$$y = (0.9912x + 0.125) \text{ mmol/l}; r = 0.997 (n = 67).$$

Measurement of serum urea with the device yielded only a poor correlation because variations of the pH and buffer capacity of the biological sample caused by proteins and bicarbonate gave rise to unsystematic deviations. The assay was improved by subtracting the signal of an enzyme-free electrode connected to another Glukometer analyzer.

5.2.3.3 Lactate

At present the determination of lactate does not belong to the most frequently performed analyses in clinical chemistry; yet its popularity in the diagnosis of shock and myocardial infarction and in neonatology and sports medicine is increasing. Strong efforts are therefore being made to develop sensor-based lactate analyzers which may be readily used at the bedside.

The normal lactate concentration in blood is between 1.2 and 2.7 mmol/l. For accurate lactate determination hemolysis of the sample is required to account for the (low) lactate content of erythrocytes. On the other hand, the glycolytic reactions in the sample have to be efficiently and rapidly inhibited in order to avoid lactate formation. Therefore the best-suited sample material is deproteinized blood; however, the time period inevitably required for its preparation prevents rapid lactate assay. That is why the study of blood lactate sensors focuses not only on the sensor itself but also on the rapid pretreatment of blood samples.

The first enzyme electrode-based lactate analyzer was developed in 1976 by La Roche (Switzerland) (see Table 23). It uses cytochrome b_2 in a tiny reaction chamber on top of a platinum electrode polarized at +0.25–0.40 V. The solution for blood sample pretreatment recommended by the manufacturer has been improved by Soutter et al. (1978) by addition of cetyltrimethylammonium bromide. This compound hemolyzes the sample, stabilizes the lactate content, and leads to a good correlation with the spectrophotometric reference method using deproteinized blood:

$$y = (1.007x + 0.024) \text{ mmol/l}; r = 0.9813 (n = 53).$$

Another prescription by La Roche involving the use of penthanil and saponin led to the following correlation (Geyssant et al. 1985):

$$y = (0.96x + 0.42) \text{ mmol/l; } r = 0.97 \text{ (} n = 88 \text{)}.$$

The analyzer has also been employed to measure lactate in muscle biopsy specimens (Denis et al., 1985).

Other lactate analyzers use lactate oxidase (LOD). Clark et al. (1984b) use the enzyme in the YSI 23L instrument (USA) as immobilized between a cellulose acetate membrane and a polycarbonate membrane, the latter serving to exclude high-molecular weight interferents. Lactate measurement in whole blood pipetted immediately after withdrawal into the phosphate buffer stream of the analyzer yielded the following correlation with values obtained with deproteinized blood (Weil et al., 1986):

$$y = (0.95x - 0.17) \text{ mmol/l; } r = 0.994 \text{ (} n = 179 \text{)}.$$

The sensor measures only plasma lactate, whereas the results of the reference method reflect the concentration of lactate in both plasma and erythrocytes. The authors found an average deviation of the sensor values of 5%. This deviation is surprisingly low in view of the high erythrocyte content of blood (hematocrit 10–50%), which should lead to significantly lower lactate values. The authors therefore postulated a uniform distribution of lactate between plasma and blood cells. Given this, however, the deviation of the values measured with the sensor should be even larger, since the real sample volume, i.e., that of plasma, would be much below 25 μl . These contradictory results provoke doubts as to the applicability of the YSI 23L and the recommended procedure to the assay of lactate in whole blood.

The selectivity of the YSI 23L has been substantially increased by replacing the cellulose acetate membrane by a cellulose acetate-butyrate membrane (Weil et al., 1986). This membrane prevents the permeation of anodically oxidizable drugs, such as paracetamol and aminoguanidine, to the electrode surface. The analyzer has been successfully used for lactate determination in spinal fluid (Clark et al., 1984a).

In the lactate analyzer HER-100 (Omron Tateisi, Japan) an asymmetric cellulose acetate membrane is used, bearing LOD covalently bound by γ -aminopropyl triethoxysilane and crosslinked by glutaraldehyde (Tsuchida et al., 1985). The membrane is highly selective for hydrogen peroxide. The analyzer has been employed for lactate assay in

human serum. Good agreement with the spectrophotometric method was found for control serum.

Weigelt et al. (1987a) adapted the Glukometer to lactate measurement by using lactate monooxygenase (LMO). Since the method is based on oxygen measurement, plasma has been used as sample material. For 30 samples the correlation coefficient with the Boehringer Monotest was 0.998.

Polyurethane-immobilized LOD is being used for whole blood lactate determination in the Glukometer as well as in the ECA 20 (ESAT 6660). Dilution of the samples with the buffer described in Section 5.2.3.1 provides both complete inhibition of glycolysis and immediate hemolysis. As is shown by the correlation equations the method is quite reliable:

GKM: $y = (1.042x - 0.023) \text{ mmol/l}; r = 0.995 (n = 70)$,

ECA (ESAT): $y = (0.994x - 0.076) \text{ mmol/l}; r = 0.992 (n = 244)$.

The lactate values are available within one minute after the withdrawal of blood from the patients.

Weigelt et al. (1987b) attempted the measurement of the lactate/pyruvate ratio in plasma by using a lactate dehydrogenase–LMO sequence electrode. The sensor was connected to a pO_2 meter and was equally sensitive for lactate and pyruvate. Determination of concentrations of both substrates in a sample requires a time period of about 3 min.

5.2.3.4 Uric Acid

Normal levels of uric acid in serum are 200–400 $\mu\text{mol/l}$. Since uric acid is a risk factor for gout and other diseases, diagnosis of hyperuricemia is increasingly important.

The Glukometer (see Table 23) has been equipped with a uricase membrane and employed for uric acid assay in serum. Satisfactory agreement with the uricase-catalase reference method was obtained; the deviation of the mean value was as low as +2.4 $\mu\text{mol/l}$. The reagent costs of the method amount to only one tenth of those required for the manual photometric one.

The UA-300 analyzer of Fuji Electric (Japan) uses a uricase membrane fixed to a hydrogen peroxide selective layer (Osawa et al., 1981). Only 20 μl of blood serum is required and a sample throughput of 50–60/h at a CV of 3% is achieved. The correlation with the uricase-catalase method is reflected by the following equation:

$y = (1.1x + 0.41) \text{ mmol/l}; r = 0.97$.

The ExAn enzyme electrode-based analyzer developed by Kulys et al. (1983) is also appropriate for the measurement of uric acid. The characteristics of the device are presented in Table 8 (Section 3.1.11).

5.2.3.5 Determination of Enzyme Activities

The measurement of enzyme activity plays a key role in clinical chemistry because increased enzyme activities in body fluids often indicate damages to the tissues and cells of certain organs. Enzyme activity determination is usually carried out by measuring the initial rate of the enzyme reaction of interest in the presence of a saturating substrate concentration.

With sensors two distinct procedures are being used:

1. The product is indicated after a defined reaction period outside the measuring cell.
2. The enzyme-catalyzed reaction is allowed to proceed in the measuring cell, the reaction rate being indicated by electronic differentiation of the current–time curve.

Cholinesterase

The activity of cholinesterase in serum reflects the anabolic capacity of the liver. Values below normal (600–1400 U/l) indicate contact with cholinesterase inhibitors such as herbicides.

Cholinesterase can be assayed by measuring the choline liberated in the enzymatic reaction by using immobilized choline oxidase. Furthermore, the direct electrochemical registration of thiocholine iodide, the product of the cholinesterase-catalyzed hydrolysis of butyrylthiocholine iodide has been used (Gruss and Scheller, 1987). The Glukometer has been adapted to this reaction system by polarizing the platinum electrode to 470 mV versus an AgI electrode in 0.1 mol/l potassium iodide. The measuring solution contains 0.5 mmol/l butyrylthiocholine iodide and the reaction is started by injection of 50 μ l serum. The formation of thiocholine iodide causes an increase in the oxidation current. After a transient phase of about 20 s the reaction rate becomes constant. In the kinetic mode a measuring value proportional to the reaction rate is obtained (see also Fig. 115). For direct monitoring of the enzyme activity the reaction product is added to calibrate the indicator electrode.

A good correlation with the standard reference method has been obtained for both serum cholinesterase and the isozyme present in erythrocytes:

$$y = (1.0108x - 4.1) \text{ U/l}; r = 0.994 (n = 27).$$

The excellent precision is demonstrated by a CV below 2%.

As modified in this manner, the Glukometer can also be employed to detect cholinesterase inhibitors. When a serum sample of known enzyme activity is used, the reaction rate decreases upon addition of an inhibitor and the remaining activity is displayed after 30 s.

This reaction system is being utilized commercially in the Bioalarm instruments of Thorn EMI (England) and Midwest Research Instruments (USA) incorporating immobilized cholinesterase.

Alanine Aminopeptidase

The determination of alanine aminopeptidase (AAP, EC 3.4.11.14) is of importance in the rapid diagnosis of liver and bile diseases. Common assays involve the coupling of alanine hydrazide cleavage with a chromogenic reaction. Kirstein (1987) proposed indicating the rate of hydrazine formation electrochemically. In contrast to an amperometric urea sensor based on this indication method (see Section 3.1.21), the pH value in the near-electrode space remains unchanged while the concentration of electrode-active hydrazine rises. The incubation period for AAP assay is lowered to half of that needed in the conventional method, the two correlating with $r = 0.994$.

α -Amylase

Increased serum activity of α -amylase indicates several internal diseases. The enzyme catalyzes the stepwise hydrolysis of starch and oligosaccharides to maltose. Since studies with α -amylase use rather heterogeneous substrates, comparison of the given activities is often impossible.

The α -amylase analyzer of Fuji Electric (Japan) (Osawa et al., 1981) is based on a GOD electrode (see Table 23). The sensor measures the endogenous glucose concentration of the sample and the rate of glucose liberation after addition of maltopentaose and α -glucosidase (maltase).

The formation of low-molecular weight products of the α -amylase-catalyzed starch hydrolysis can be assayed by using a glucoamylase-GOD electrode (Pfeiffer et al., 1980). The sensor is covered by a dialysis membrane with a cutoff of 15 kDa which prevents starch from reaching the enzymes. The cleavage products can easily diffuse into the bienzyme membrane where they are successively degraded to glucose by glucoamylase. As only the β -anomer is formed, the sensitivity of the method

is higher than with α -glucosidase. Litschko (1988) optimized the procedure by completely removing the endogenous glucose during the incubation period with GOD. However, it requires 10 min to process a sample; the method therefore is only useful for discrete analysis. It correlates with the iodine-starch method as follows:

$$y = (1.077x - 0.998) \text{ U/l}; r = 0.947 (n = 21).$$

Lactate Dehydrogenase

LDH is a tetrameric enzyme occurring in several isozymes. Knowledge of the total activity of LDH in serum is important for the differential diagnosis of heart and liver diseases and pernicious anemia. The normal levels are up to 240 U/l.

In principle, lactate analyzers such as the YSI 23L and the ECA 20 are applicable to LDH assay after incubation of the serum sample with NADH and pyruvate. In such methods the endogenous lactate has to be either removed or measured before LDH measurement can be carried out. The latter has been studied by Mizutani et al. (1982) and Weigelt et al. (1987b). When the sensor indicates a stable lactate value the LDH reaction is initiated by injection of substrate. The subsequent current increase is linear within a certain time period and is related to the enzyme activity. Mizutani et al. used a lactate oxidase sensor and were able to detect LDH between 138 and 414 U/l with a correlation coefficient with the photometric method of 0.995. A linear measuring range up to 1200 U/l has been obtained by using a lactate monooxygenase electrode (Weigelt et al.). The CV of 20 determinations of a serum sample containing 252 U/l was 1.2%. The correlation was:

$$y = (1.11x - 17.4) \text{ U/l}; r = 0.999 (n = 30).$$

The procedure permits 15–20 combined measurements of lactate and LDH per hour to be carried out.

Pyruvate Kinase

The clinical importance of pyruvate kinase lies in the diagnosis of pyruvate kinase deficiency in erythrocytes, which is the second most common congenital enzyme defect and leads to chronic hemolytic anemia.

Pyruvate kinase (PK) activity in hemolyzed erythrocytes has been determined by using an LDH-lactate monooxygenase sequence electrode (Weigelt et al., 1988). The enzymes were immobilized in gelatin and attached to an oxygen probe. Since the sample material contains only

negligible amounts of lactate and pyruvate, the pyruvate kinase activity can be derived directly from the current decrease on addition of the PK substrates, phosphoenolpyruvate and ADP, and the LDH cofactor, NADH. Calibration was performed by using a standard pyruvate solution. The measuring time was about 4 min. The relative standard deviation for 6.6 units of pyruvate kinase per gram of hemoglobin was 3.1%; good agreement with the spectrophotometric method was found.

Since the sensor signal was linear over the normal range of 2.1–6.9 U/g hemoglobin, and only decreased activities are of clinical relevance, the bienzyme electrode is suitable for the measurement of any clinically possible value.

Transaminases

The determination of alanine aminotransferase and aspartate aminotransferase (ALAT and ASAT, formerly GPT and GOT) in clinical chemistry is as important as that of glucose. The normal ranges are between 5 and 24 U/l for ALAT and between 5 and 20 U/l for ASAT. The enzyme activities can rise up to 1000-fold in acute hepatitis, myocardial infarction, or alcoholic insult.

The reactions of ALAT and ASAT can be monitored by sensing their products, pyruvate, oxaloacetate, and glutamate, with enzyme electrodes. Kihara et al. (1984b) developed a bienzyme electrode for the sequential determination of both transaminases. The sensor was composed of oxaloacetate decarboxylase and pyruvate oxidase adsorbed on a PVC membrane, and a hydrogen peroxide-indicating electrode. The sequential determination was carried out as follows: the sample was added to a measuring solution containing α -ketoglutarate. After addition of aspartate the current–time curve increased linearly with time. Another substrate solution including alanine was fed into the cell and a further increase of the current–time curve was observed. The slopes of the two straight lines were proportional to activity up to 1500 U/l for both enzymes. The time period required for one sequential measurement was 4 min. The correlation coefficient between the sensor and the optical method was 0.99.

Employment of glutamate oxidase enables the sequential assay of both transaminases without the need to coimmobilize a second enzyme. Using a glutamate oxidase sensor, Yamauchi et al. (1984) found a preincubation of the sample of 30 min to be necessary. The preincubation time could be lowered to 10 min by using an optimized sensor configuration (Wollenberger et al., 1989).

5.3 CONTINUOUS PATIENT MONITORING AND IMPLANTABLE SENSORS

5.3.1 *Monitoring of Blood Glucose*

For the monitoring of diabetics during stressful situations such as surgery, traumata, or myocardial infarction, glucose-controlled insulin infusion systems are required. Pathophysiological mechanisms that tend to increase the insulin demand in an unpredictable manner can lead to life-threatening states of the organism. Euglycemia should therefore be adjusted during and after stress situations.

Battery-driven insulin pumps of the size of a pocket calculator with a subcutaneous injection needle have yet been developed. Though microprocessor-controlled, they are unable to exclude hypoglycemic states when extreme deviations occur. Knowledge of the current glucose level is indispensable for optimal insulin dosing. Up to now, alternative physiological parameters are unknown.

For about 15 years, research has been conducted on the development of implantable glucose sensors for continuous monitoring of the blood glucose level. An artificial β -cell for perioperative glucose monitoring, the Biostator, has been commercialized by Life Science Instruments (USA) (Fogt et al., 1978). The equipment consists of an on-line glucose analyzer based on a H_2O_2 -detecting GOD electrode which is integrated in a computer-controlled feedback system and can be used for 24–48 h. The system permits dynamic blood glucose control but has not been generally accepted in routine clinical applications due to the number of personnel required, the limited stability of the glucose sensor, and the possibility of erroneous calculation of the insulin dosage.

An alternative approach has been devised for perioperative monitoring of diabetics (Kiesewetter et al., 1985; Scheller et al., 1986b). The monitoring system, named Glucon, is based on a modified Glukometer analyzer (see Section 5.2.3) coupled with a computer for dialogue-oriented control, and infusion pumps for insulin and glucose. The control algorithm uses the forecast of the glucose concentration derived from glucose and insulin infusion rates of the foregoing 70 minutes by means of an internal dynamic glucose model. The infusion rates, insulin action factor, and time course of the glucose concentration are recorded and are thus readily available to the physician.

The *in vivo* application of glucose sensors is restricted by immunological reactions of the organism against the implanted material. The

biocompatibility of the materials to be used, above all enzyme membranes, is thus being studied. Mullen (1986) proposed covering a needle-type glucose sensor with a silanized membrane in order to increase the biocompatibility of the probe. As judged by scanning electron microscopy, deposition of protein on the membrane was less drastic in tissue than in the bloodstream (Vadgama, 1986).

Another problem with the development of implantable sensors is the need to calibrate the sensor *ex vivo*. This requires a high enzyme stability since the sensor has to be calibrated before implantation. The longest lifetime reported for enzymes immobilized in an implanted sensor was between 6 and 10 days (Shichiri et al., 1987). Neither physical entrapment nor chemical binding and crosslinking of GOD have provided a higher stability for continuously operated glucose sensors.

The physiological oxygen concentrations in arterial blood, 0.15 mmol/l, and venous blood, 0.01 mmol/l, are much lower than that of glucose (5–15 mmol/l). Continuous flow-through blood glucose sensors based on oxygen probes therefore exhibit a nonlinear current-concentration dependence (Layne et al., 1976).

Kessler et al. (1984) developed a glucose sensor with an extremely low oxygen demand and a stability of 3 months, which appears to be suitable for implantation. Another sensor that might be implantable is based on the use of ferrocene as an electron acceptor for GOD (Cass et al., 1984; David et al., 1985), which eliminates the need for oxygen. The sensor exhibits an advantageous linear range of 1–30 mmol/l. However, experiments with the sensor implanted subcutaneously in animals revealed a rapid sensitivity decrease (Pickup, 1987).

A glucose sensor system consisting of an oxygen electrode and a glucose oxidase electrode was recently implanted into the *vena cava* of a dog (McKean and Gough, 1988). The sensors operated in the potentiostatic mode and were connected to an implantable telemetry system. Both the enzyme stability and the power consumption allowed operation of the system for three months.

Abel et al. (1984) succeeded in expanding the linear range of a glucose electrode up to 40 mmol/l by covering the enzyme membrane with a perforated hydrophobic polyethylene membrane. As applied subcutaneously and with a pO_2 of 2–5 kPa the sensor was stable for some hours (Fig. 134).

Similarly to the above authors, Shichiri et al. (1984) introduced a glucose sensor into interstitial fluid. In animal experiments a measuring range of 3.3–27.7 mmol/l was obtained. Combination of a cellulose

diacetate membrane containing GOD with a hydrophilic alginate–polylysine–alginate membrane provided a response time of 5 min and a stability of 7 days. In contrast, with a poly(vinyl alcohol)–GOD membrane the lifetime was only 3 days. The increased lifetime of the sandwich membrane was claimed to be due to better biocompatibility (Shichiri, 1987).

The studies by Shichiri's group led to an artificial pancreas consisting of a needle-type glucose sensor, a computer, and two syringe-driving systems, with a total weight of 400 g. Yet years will have to pass until a robust and reliable equipment for everyday use will be available.

5.3.2 Urea Determination in the Artificial Kidney

The continuous measurement of urea in blood during dialysis is an important precondition for optimization and individual monitoring of the treatment of patients with chronic kidney failure. The methods of urea quantification employed so far are time-consuming and have to be

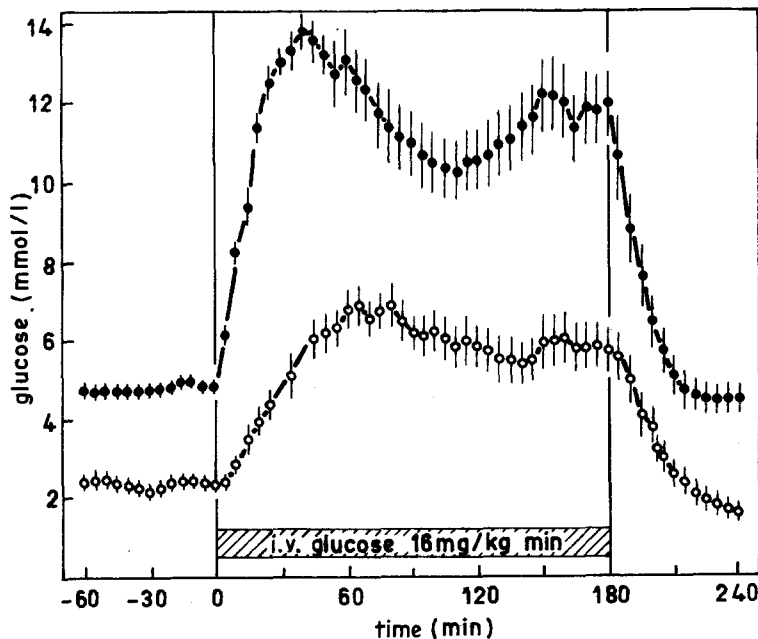


Fig. 134. Time course of the concentration of glucose in periphero-venous plasma (●) and of the signals measured with a glucose electrode in subcutaneous tissue (○) of a healthy dog intravenously infused with glucose. (Redrawn from Müller et al., 1986).

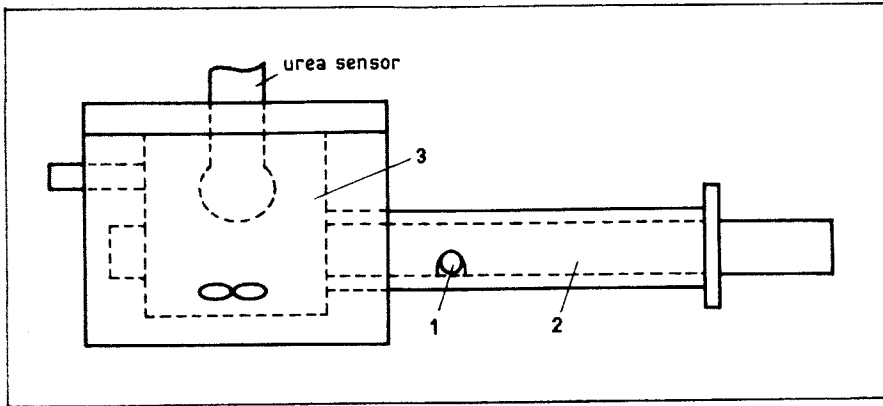


Fig. 135. Measuring cell of the urea module with dialyzate sampler. The sample is transferred in the hole (1) of the movable piston (2) from the dialyzate cycle into the measuring cell (3).

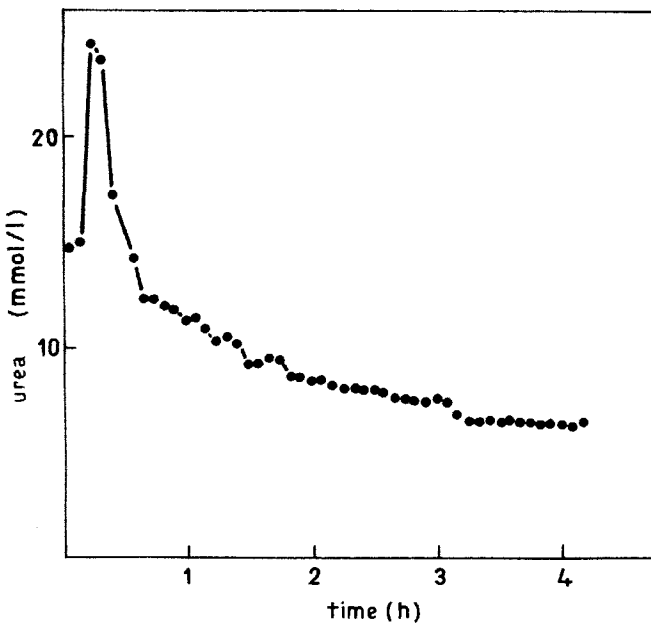


Fig. 136. Urea determination with the urea module during dialysis treatment of a patient.

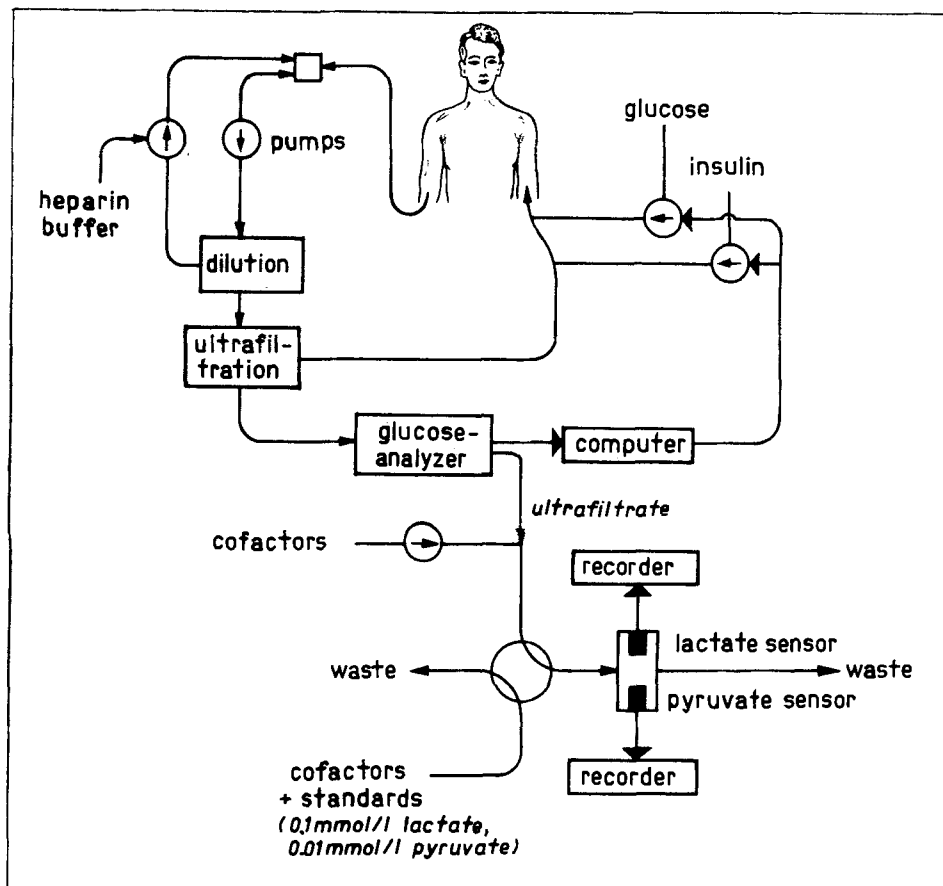


Fig. 137. Scheme of an on-line bedside analyzer for parallel determination of glucose, lactate, and pyruvate using enzyme electrodes. (Redrawn from Mascini, 1987).

performed in the laboratory. The results are generally available only after dialysis.

The determination of urea in dialyzate is methodologically superior to the determination in whole blood. The blood and tissue urea concentrations can easily be calculated from the values obtained by using a simple mathematical model. Scheller et al. (1986b) developed an enzyme electrode for the semicontinuous assay of urea in dialyzate and integrated the sensor into an artificial kidney. The electrode is inserted in a flow-through cell (Fig. 135) that is hydraulically connected to the hemodialyzer. Control of the measuring cycle and signal processing is conducted electronically. The urea unit allows 20 measurements per hour to be carried out, thus providing sufficiently exact monitoring of

the time course of urea concentration. The enzyme electrode is stable for more than 1 week when used in the hemodialyzer for about 4 hours per day. The CV obtained for measurement of urea in dialyzate was about 3% and the correlation coefficient with the reference method was 0.99.

Practical use of this unit permits direct control of the efficiency of hemodialysis treatment (Fig. 136) which can thus be optimally adapted to the patient's needs. In the future it should be possible to affect the relevant medical treatment parameters on the basis of the measured urea concentration values.

5.3.3 Determination of Lactate and Pyruvate

An Italian research group (Mascini, 1987; Mascini et al., 1987) introduced three enzyme electrodes, namely for the determination of lactate, pyruvate, and glucose, into the 'Betalike' artificial pancreas (Elettronica Esacontrol, Genova) (Fig. 137). The patient's blood was dialyzed and pumped through the measuring cells containing the sensors. For the glucose and lactate sensors, oxygen probes were used whereas the low pyruvate concentration in blood (40–120 $\mu\text{mol/l}$) required the use of a hydrogen peroxide electrode for assembling the pyruvate sensor. The set-up enables monitoring of the three substrate concentrations during the treatment of diabetics. The values obtained within a treatment period of 16 h agreed satisfactorily with those measured by reference methods.

5.4 FOOD ANALYSIS, BIOPROCESS CONTROL AND ENVIRONMENTAL MONITORING

The quality assessment of food and fodder products requires analysis of protein, carbohydrates and fat. The enzyme electrode-based analyzers originally developed for clinical chemistry have found only limited application in food analysis because they are only suitable for the determination of one parameter, mostly glucose or a disaccharide. The increasing concern for food quality require new types of biosensors allowing residual and hygiene control and on-line measurement of age and freshness (Tschannen, 1988).

A peculiarity of food analysis is the presence of large amounts of potentially interfering compounds in many food stuffs. By using the Glukometer (see Section 5.2.3) for assaying glucose and sucrose in instant drinks, Scheller and Karsten (1983) found considerable interfer

ences by the high concentrations of ascorbic acid and vanilline in the samples. Such samples therefore have to be assayed by means of oxygen rather than hydrogen peroxide probes. The samples must be carefully saturated with air prior to the measurement.

Weise et al. (1987) proposed saturating food and fermentation samples by bubbling with air directly in the reaction vessel of a modified automated sampler. During the oxygenation, hydrolysis of the analytes sucrose, glucosinolate, or starch was performed. Up to 60 samples per hour could be analyzed automatically with good precision.

Enzyme sensors involving hydrogen peroxide-sensing electrodes can be readily employed for the determination of sucrose in sugar beet juice and of lactose in milk. The Enzyme-Chemical Analyzer ECA 20 (Prüfgeräte-Werk Medingen, GDR) as furnished with a β -galactosidase-GOD electrode is capable of analyzing the lactose content of up to 100 milk samples per hour with a CV below 2%.

In the Glucoprocasseur (Tacussell, France), interference by reducing substances is compensated for by using a difference measurement technique. The analyzer has been used for assaying glucose, lactate, and oxalate in foodstuffs (Coulet, 1987). The alcohol analyzer from YSI (USA) is applicable to the analysis of hard liquor. The enzyme membrane used has a poor working stability, however.

In biotechnological processes effective exploitation of raw materials and high yields can be gained by process control involving on-line determination of a multitude of parameters. In fermentation processes, e.g., of antibiotics, a certain time course of the concentration of such nutrients as carbohydrates, amino acids, phosphates, and ammonium, as well as hormones has to be followed closely. The determination of these substances is therefore an indispensable prerequisite for optimization of the product yield. Knowledge of the product concentration permits direct evaluation of the state of the bioprocess. Table 25 gives an overview of the value of biotechnological products and points to the potential areas of biosensor application in fermentation and cell culture media.

Environmental protection requires an ever-increasing arsenal of analytical methods to assess the quality of soil, water and air. The most prominent analytes in this area are organic wastes, heavy metals and toxic gases.

Enzyme electrodes have been described for the majority of the low-molecular substances of interest, such as amino acids, sugars, phosphates, penicillin and gluconic acid. In contrast, fundamental problems

TABLE 25

1982 World Market for Biotechnological Products (Bakker et al., 1984)

	Million US \$
Ethanol	500
Glutamic acid	500
Citric acid	300
Gluconic acid	35
Single cell protein	600
Enzymes	1000
Antibiotics	8000
Insulin	310
Monoclonal antibodies	4000

have to be faced in the sensor-based determination of high-molecular weight compounds like enzymes and antibodies. Finally, the application of biosensors of any kind in fermenters is associated with significant difficulties:

- (i) direct sterilization of biosensors is impossible;
- (ii) discrete measurements are necessary for calibration of the sensor;
- (iii) in most cases the analyte concentration exceeds the linear range of the sensor;
- (iv) various interfering substances have to be expected;
- (v) the sensor's stability is affected by mechanical and thermal stress.

Enfors (1982) employed an oxygen-stabilized enzyme electrode for glucose monitoring in a batch culture of *Candida utilis*. Agreement with a reference method was fairly good. As expected, the concentration of glucose decreased with increasing cell growth. No disturbances by variations of the oxygen partial pressure during fermentation or by undesired electrochemical reactions were observed.

The measuring range of a glucose sensor has been expanded by coupling of GOD or glucose dehydrogenase to a ferrocene-modified electrode (Turner, 1985). Brooks et al. (1987/88) significantly enhanced the lifetime of this sensor by immobilizing GOD covalently to alkylamine groups on the electrode surface. The sensor was introduced into a fermenter via a sterilizable housing through which buffer was continu-

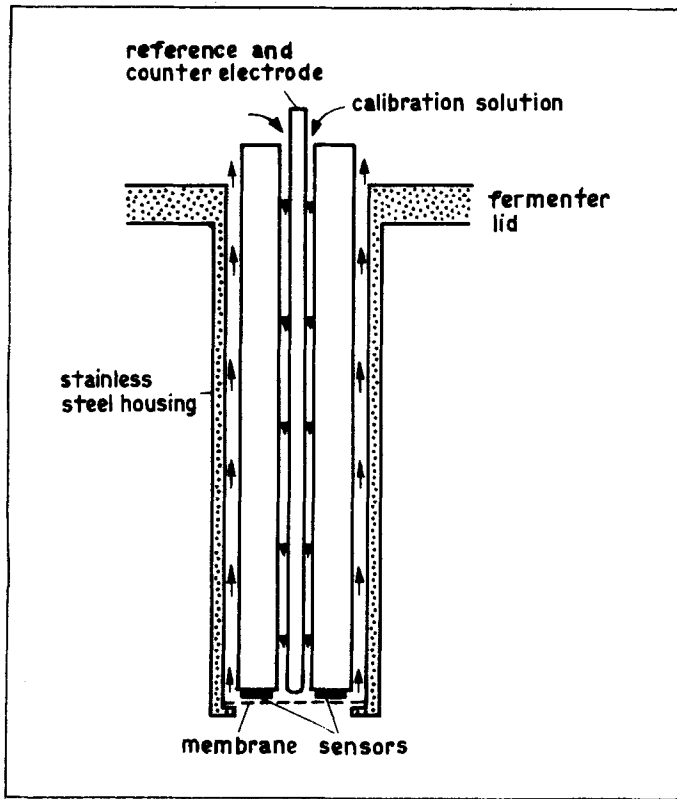


Fig. 138. *In-situ* glucose sensor for fermentation control. (Redrawn from Brooks et al., 1987/88).

ously pumped (Fig. 138). The housing was separated from the fermentation broth by a polycarbonate membrane of $0.22\ \mu\text{m}$ thickness. The usable, but nonlinear measuring range of the sensor reached up to $100\ \text{mmol/l}$; the lifetime was 14 days. For application in an *E. coli* batch culture the baseline and sensitivity of the electrode had to be corrected.

A sensor system involving an alcohol oxidase electrode and an enzyme-free oxygen probe has been used for continuous assay of ethanol in alcoholic fermentation (Verduyn et al., 1984). The bare O_2 electrode served to compensate for $p\text{O}_2$ variations in the fermenter. The measuring range was rather narrow so that only the initial phase of ethanol formation could be followed.

These examples indicate the *in situ* applicability of enzyme electrodes; yet numerous problems have to be solved. At present, coupling of enzyme sensors for fermentation control in a bypass arrangement

appears to be more favorable (Klopper et al., 1989). Following this concept, Mandenius et al. (1981) developed an invertase thermistor incorporating a sterilizable filter unit. The equipment has been employed to monitor alcoholic fermentation by immobilized yeast cells. A thermistor has been successfully used for on-line glucose measurement under real cultivation conditions of *Cephalosporium acremonium* (Wehnert et al., 1987). Similar calorimetric devices have been used in other fermentation processes and in environmental analysis (Table 26).

TABLE 26

Application of Enzyme Thermistors in Process Control and Environmental Monitoring

Analyte	Enzymes	Measuring range (mmol/l)
Cellobiose	β -glucosidase + glucose oxidase + catalase	0.05–5
Cephalosporin	cephalosporinase	0.005–10
Ethanol	alcohol oxidase	0.01–1
Galactose	galactose oxidase	0.01–1
Lactose	β -galactosidase + glucose oxidase + catalase	0.05–10
Penicillin G	β -lactamase	0.01–500
Sucrose	invertase	0.05–100
Heavy metal ions (e.g. Pb^{2+})	urease	10^{-9}
Insecticides (e.g. parathion)	acetylcholinesterase	$5 \cdot 10^{-6}$
Cyanide	rhodanase	0.02–1
Phenol	tyrosinase	0.01–1

Geppert and Asperger (1987) employed an enzyme electrode to control the concentration of glucose in various bioprocesses. The samples were continuously withdrawn, deaerated with nitrogen, and diluted, without separation of biomass. Glucose was analyzed by discrete measurement with the use of the artificial electron acceptor benzoquinone. The method required correction of the measurement by means of an enzyme-free sensor.

Process control in human and animal cell culture is extremely important because the required nutrients, e.g. fetal calf serum, are extraordinarily expensive (Merten et al., 1987). Tsuchida et al. (1985) employed the lactate analyzer HER-100 (Omron Tateisi, Japan) together with a glucose electrode for lactate and glucose analysis in the growth medium of human melanoma cells. Investigation of the process over 7 days

showed that, as a result of glycolysis, the concentration of glucose decreased with increasing cell number and that of lactate increased. No reference determinations were carried out.

The possibility of withdrawing representative samples without affecting the sterility of the bioreactor is an essential prerequisite for on-line monitoring. Appropriate filtration equipment is being offered by Braun Melsungen (FRG) and Control Equipment (USA). A mechanical sampling system automatically leading the sample through a sterilized chamber before dilution has been designed at the Massachusetts Institute of Technology (USA). The equipment has been combined with the Enzymat analyzer (Seres, France) in order to monitor the production of monoclonal antibodies against fibronectin by hybridoma cells (Fig. 139) (Romette, 1987). Every 30 minutes the concentrations of glucose, lactate, and glutamine were measured in parallel. The enzymes were contained in glutaraldehyde-crosslinked gelatin membranes in front of oxygen electrodes. The sensor for glutamine determination comprised coimmobilized glutaminase from *E. coli* and glutamate oxidase from *Streptomyces* sp. The measured data permitted the derivation of a relationship between the substrate concentrations, ATP flux, and cell growth.

The glucose electrode of YSI is being used as the sensor in an analyzer

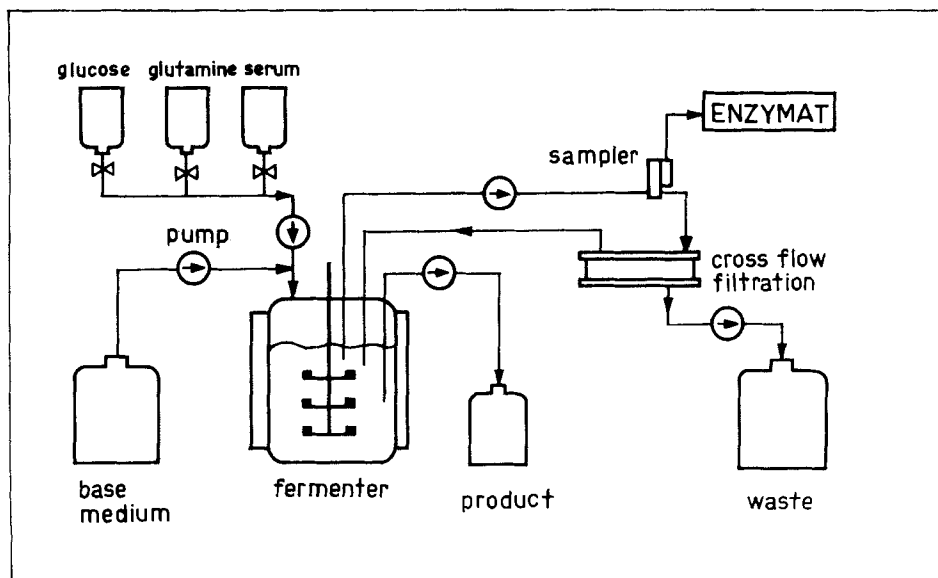


Fig. 139. Sensor-controlled cell cultivation system. (Redrawn from Romette et al., 1987).

for cell cultivators from Control Equipment. The sample stream is separated from the biomass by cross filtration and is periodically introduced into an FIA instrument. Since H_2O_2 is indicated, the sensor signals are affected by interference from various components of the culture medium.

The On Line Biotec Analyzer PM-1000 for automatic process control in industrial fermentation and biotechnology research is being sold by Nippon General Trading Co. (Japan). The instrument combines the enzyme electrodes contained in the laboratory analyzers M 100, AS-200 and AD-300 (Toyo Jozo, Japan) with a unit for sterile filtration and a computer. By exchanging the enzyme electrodes the concentrations of glucose, ethanol, L-lactate, glycerol, sucrose, lactose, pyruvate, ascorbic acid, or L-amino acids can be monitored. The sensors use the appropriate oxidases and, in addition, glycerokinase for glycerol determination and β -galactosidase for lactose determination. For sucrose assay, invertase is combined with pyranose oxidase in order to avoid disturbances by endogenous sample glucose. Since oxygen probes are used as base sensors, problems may be caused by varying O_2 concentrations in the fermentation broths.

Microbial sensors are being routinely used for the analysis of effluent water in Japan (Karube, 1986). They indicate the wastewater constituents that are assimilable by microbes, i.e., a parameter similar to the biological oxygen demand (BOD).

A conventional BOD determination requires 5 days and is thus unsuitable for process control. Sensors for rapid BOD estimation have therefore been developed by using immobilized cells of *Bacillus subtilis* and *Trichosporon cutaneum* (Riedel et al., 1987). They measure the acceleration of respiration resulting from nutrient supply, i.e., no steady state has to be reached. The sensor is calibrated in a solution containing equimolar concentrations of glucose and glutamic acid. The signal depends linearly on concentration up to 100 mg/l. The least detectable concentration is 4 mg/l. The short measuring time makes the sensor highly suitable for the monitoring of wastewater treatment.

A limitation to this approach is that the organic waste water components are converted with different reaction velocities. Macromolecules, such as starch and proteins, are not indicated at all. This might be overcome by enzymatic sample pretreatment or by the use of hybrid sensors.

Chapter 6

Perspectives — Combination of Biotechnology and Microelectronics in Biosensors

Biosensors are the offspring of the first successful marriage between biotechnology and modern electronics. The biomolecules are responsible for the specific recognition of the analyte whereas the physicochemical transducer supplies an electrical output signal which is amplified by the spatially separated electronic component.

The traditional concept of internal signal processing in sensors originates from the development of chemically sensitive field effect transistors. The first step in this development has been the inclusion of a transistor in the electrode body of ion selective electrodes. The direct coverage of electronic devices by ion sensitive or biologically active layers to form enzyme- or immunoFETs leads to the integration of (bio)specific recognition and electronic signal processing. When such devices are used, disturbances can be excluded by difference measurement and statistical evaluation by multigate sensors becomes feasible. Biosensors with internal electronic signal processing can be advantageously employed to control integrated actuators, e.g., insulin or drug delivery systems. The whole system is then a closed loop receptor-actuator unit quite analogous to biological sensory organs.

Internal signal processing in biosensors can be realized by coupled enzyme reactions at the stage of molecular recognition. An important aspect of the inherent signal processing affected by coupled reactions with enzymes is the analogy to mathematical operations (Fig. 140) (Scheller et al., 1985b). A sensor containing two independent enzymes or an enzyme sequence with identical sensitivities carries out the *addition* of the signals of the two respective monoenzyme sensors. The signal of a competition sensor of two enzymes for one substrate is equivalent

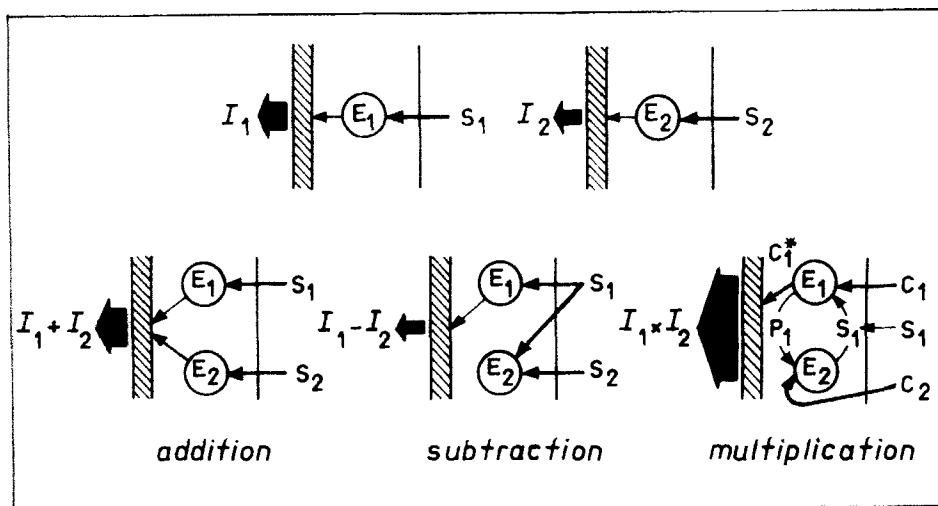


Fig. 140. Internal signal processing in biosensors using coupled enzyme reactions. (Redrawn from Scheller et al., 1985b).

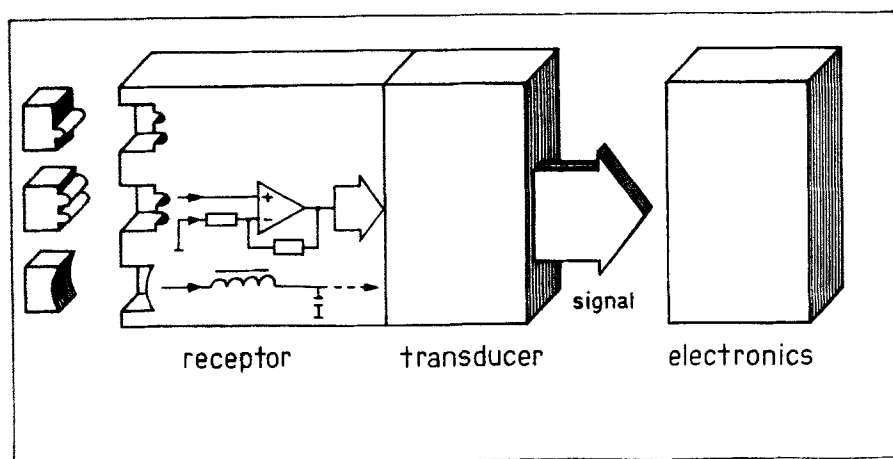


Fig. 141. Coupling of molecular recognition and internal signal processing, e.g. amplification and chemical filtering, by coupled enzyme reactions in biosensors.

to the *difference* of the signal of the substrate enzyme sensor and that of a cofactor-indicating sensor. Enzymatic substrate recycling effects the *multiplication* of the signals of the respective monoenzyme electrodes. In substrate elimination by an anti-interference membrane the limited

capacity of the eliminator enzyme causes a threshold value of concentration above which a signal is indicated. The sensor therefore acts as a *threshold switch*.

Consequently, coupled enzyme reactions may serve to amplify the measuring signal or to chemically filter disturbing substances (Fig. 141). This kind of signal processing mimics functions that are traditionally performed by electronics and thus significantly increases the analytical potential of biosensors. At present the use of coupling principles — particularly cycling reactions — in *biocomputers* is being discussed (Okamoto et al., 1987).

Coupled enzyme reactions in biosensors are modeled on natural metabolic pathways. The evolution-optimized arrangement of the interacting parts of these pathways renders them significantly more efficient than the corresponding isolated enzyme systems in solution. The biotechnological simulation of this arrangement in site-to-site oriented immobilized enzymes (Mansson et al., 1983) and fused multisite enzymes (Bülow and Mosbach, 1987) may provide powerful receptor systems for molecular recognition and internal signal processing in biosensors. Future research will focus on the combination of biological and electronic signal processing. This development will necessarily involve close cooperation between scientists and engineers from different fields of the technological spectrum; a truly multidisciplinary opportunity.

This Page Intentionally Left Blank

List of Abbreviations and Symbols

ADP	adenosine diphosphate
AFP	α -fetoprotein
ab	antibody
ag	antigen
ALAT	alanine aminotranferase
AP	alkaline phosphatase
ASAT	aspartate aminotransferase
ATP	adenosine triphosphate
BQ	benzoquinone
BSA	bovine serum albumin
CEH	cholesterol ester hydrolase
CK	creatine kinase
CME	chemically modified electrode
COD	cholesterol oxidase
con A	concanavalin A
CV	coefficient of variation (relative standard deviation)
<i>d</i>	layer thickness
<i>D</i>	diffusion coefficient
E	enzyme
<i>E</i>	potential
EC	Enzyme Classification
ECME	enzyme-chemically modified electrode
EDTA	ethylene diamine tetraacetic acid
EIA	enzyme immunoassay
f_E	enzyme loading factor
FAD	flavin adenine dinucleotide
FET	field effect transistor
FIA	flow injection analysis
<i>G</i>	amplification factor
GOD	glucose oxidase
G6P-DH	glucose-6-phosphate dehydrogenase
HB _s	hepatitis B surface antigen
HCG	human chorionic gonadotropin

HIS	higher integrated biocatalytic system
HK	hexokinase
H ₂ Q	hydroquinone
HSA	human serum albumin
HRP	horseradish peroxidase
<i>I</i>	current
IgG	immunoglobulin G
ISE	ion selective electrode
ISFET	ion sensitive field effect transistor
<i>K_M</i>	Michaelis constant
LDH	lactate dehydrogenase
LMO	lactate monooxygenase
LOD	lactate oxidase
<i>M</i>	mediator
MCME	mediator-chemically modified electrode
MOS	metal oxide semiconductor
MV	methylviologen
NAD ⁺	nicotinamide adenine dinucleotide, oxidized
NADH	nicotinamide adenine dinucleotide, reduced
NADP ⁺	nicotinamide adenine dinucleotide phosphate, oxidized
NADPH	nicotinamide adenine dinucleotide phosphate, reduced
NMP ⁺	N-methylphenazinium
<i>P</i>	product
<i>P</i>	product concentration
PK	pyruvate kinase
PVA	poly(vinyl alcohol)
PVC	poly(vinyl chloride)
R	receptor, chemically sensitive reagent
RIA	radioimmunoassay
<i>S</i>	substrate
<i>S</i>	substrate concentration
SCE	saturated calomel electrode
TCNQ	tetracyano-p-quinodimethane
<i>v_{max}</i>	maximum rate of enzyme reaction
<i>V</i>	voltage

References

- Abel, P., Müller, A., and Fischer, U. (1984). *Biomed. Biochim. Acta* **43**, 577
- Adam, G., Länger, P., and Stark, G. (1977). *Physikalische Chemie und Biophysik*, Springer, Berlin
- Ahern, T.J., and Klibanov, A.M. (1986). In: *Protein Structure, Folding and Design* (Oxender, D.L., Ed.). Alan R. Liss Publishers, New York, p. 283
- Ahn, B.K., Wolfson, S.K., and Yao, S.J. (1975). *Bioelectrochem. Bioenerg.* **2**, 142
- Aizawa, M. (1982). *Denki Kagaku* **50**, 981
- Aizawa, M. (1983). *Proc. Int. Meeting Chemical Sensors*, Fukuoka, Elsevier, Amsterdam, p. 683
- Aizawa, M., Kato, S., and Suzuki, S. (1977). *J. Membrane Sci.* **2**, 125
- Aizawa, M., Morioka, A., and Suzuki, S. (1978). *J. Membrane Sci.* **4**, 221
- Aizawa, M., Morioka, A., Suzuki, S., and Nagamura, Y. (1979a). *Anal. Biochem.* **94**, 22
- Aizawa, M., Suzuki, S., Nagamura, Y., Shinohara, R., and Ishiguro, J. (1979b). *J. Solid-Phase Biochem.* **4**, 25
- Aizawa, M., Kato, S., and Suzuki, S. (1980a). *J. Membrane Sci.* **7**, 1
- Aizawa, M., Morioka, A., and Suzuki, S. (1980b). *Anal. Chim. Acta* **115**, 61
- Aizawa, M., Wada, M., Kato, S., and Suzuki, S. (1980c). *Biotechnol. Bioeng.* **22**, 1769
- Aizawa, M., Ikariyama, Y., and Toyoshima, T. (1983). *Denki Kagaku* **51**, 105
- Alam, I.A., and Christian, G.D. (1982). *Anal. Lett.* **15**, 1449
- Alam, I.A., and Christian, G.D. (1984). *Fres. Z. Anal. Chem.* **318**, 33
- Alam, I.A., and Christian, G.D. (1985). *Fres. Z. Anal. Chem.* **320**, 281
- Albery, W.J., and Barron, P. (1982). *J. Electroanal. Chem.* **138**, 79
- Albery, W.J., and Bartlett, P. (1985). *J. Electroanal. Chem.* **194**, 211, 223
- Albery, W.J., Bartlett, P., Craston, D., and Hagget, B. (1985). *World Biotech Rep.* **1**, 359
- Albery, W.J., Bartlett, P., and Cass, A.E.G. (1987a). *Phil. Trans. R. Soc. London* **316B**, 107
- Albery, W.J., Bartlett, P., Cass, A.E.G., and Sim, R. (1987b). *J. Electroanal. Chem.* **218**, 127
- Albery, W.J., Bartlett, P., Bycroft, M., Craston, D., and Driscoll, B. (1987c). *J. Electrochem. Soc.* **218**, 119
- Al-Hitti, I.K., Moody, G.J., and Thomas, J.D.R. (1984). *Analyst* **109**, 1205
- Anfält, T., Granelli, A., and Jagner, D. (1973). *Anal. Lett.* **6**, 969
- Anzai, J., and Hashimoto, J. (1988). *Anal. Sci.* **4**, 247
- Anzai, J., Ohki, Y., Osa, T., Nakajima, H., and Matsuo, T. (1985). *Chem. Pharm. Bull.* **33**, 2356
- Anzai, J., Furuya, K., Chen, C., Osa, T., and Matsuo, T. (1987). *Anal. Sci.* **3**, 271
- Appelqvist, R., Marko-Varga, G., Gorton, L., Torstensson, A. and Johansson, G. (1985). *Anal. Chim. Acta* **169**, 237

- Araki, T., Hara, H., and Katsube, T. (1985). *Proc. 4th Meeting Chemical Sensors Electrochem. Soc. Japan*, p. 6
- Arnold, M.A. (1985). *Anal. Chem.* **57**, 565
- Arnold, M.A. (1987). *GBF Monographs* **10**, 223
- Arnold, M.A., and Glaizer, S.A. (1984). *Biotechnol. Lett.* **6**, 313
- Arnold, M.A., and Rechnitz, G.A. (1980a). *Anal. Chem.* **52**, 1170
- Arnold, M.A., and Rechnitz, G.A. (1980b). *Anal. Chim. Acta* **113**, 351
- Arnold, M.A., and Rechnitz, G.A. (1981). *Anal. Chem.* **53**, 1837
- Arnold, M.A., and Rechnitz, G.A. (1982). *Anal. Chem.* **54**, 777
- Arwin, H., and Lundström, I. (1985). *Anal. Biochem.* **145**, 106
- Arzneibuch der DDR, Diagnostische Laboratoriumsmethoden (1976). Akademie-Verlag Berlin
- Asouzu, M.U., Nonidez, W.K., and Ho, M.H. (1990). *Anal. Chem.* **62**, 708
- Assolant-Vinet, C., and Coulet, P.R. (1986). *Anal. Lett.* **19**, 875
- Aston, W., Ashby, R., Higgins, I.J., Scott, L., and Turner, A.P.F. (1984). In: *Charge and Field Effects in Biosystems* (Allen, M.J., and Usherwood, P.N.R., Eds.), Abacus Press, London, p. 491
- Bakker, H. (1984). *Proc. 3rd Eur. Congr. Biotechnology, München, Verlag Chemie, Weinheim*, vol. IV, p. 27
- Bardeletti, G., Sechaud, F., and Coulet, P.R. (1986). *Anal. Chim. Acta* **187**, 47
- Belli, S.L., and Rechnitz, G.A. (1986). *Anal. Lett.* **19**, 403
- Bennetto, H.P., De Keyser, D.R., Delaney, G.M., Koshy, A., Mason, J., Mourle, G., Razack, L.A., Stirling, L., Thurston, C.F., Anderson, D.J., and Mullen, W.H. (1988). *Int. Ind. Biotechnol.* **8**, 6
- Bergel, A., and Comtat, M. (1984). *Anal. Chem.* **56**, 2904
- Bergveld, P. (1970). *IEEE Trans. Biomed. Eng.* **BME-19**, 70
- Bergveld, P., van der Schoot, B.H., van den Berg, A., and Schasfoort, R.B.M. (1987). *GBF Monographs* **10**, 165
- Bertermann, K., Scheller, F., Pfeiffer, D., Jänchen, M., and Lutter, J. (1981). *Z. Med. Lab.-Diagn.* **22**, 83
- Bertran, J.F. (1967). *Int. Sugar J.* **69**, 107
- Bertrand, C., Coulet, P.R., and Gautheron, D.C. (1979). *Anal. Lett.* **12**, 1477
- Bertrand, C., Coulet, P.R., and Gautheron, D.C. (1981). *Anal. Chim. Acta* **126**, 23
- Birnbaum, S., Bülow, L., Hardy, K., Danielsson, B., and Mosbach, K. (1986). *Anal. Biochem.* **158**, 12
- Blaedel, W.J., and Jenkins, R.A. (1976). *Anal. Chem.* **48**, 1240
- Blaedel, W.J., Kissel, T., and Boguslaski, R. (1972). *Anal. Chem.* **44**, 2030
- Boitieux, J.L., Desmet, G., and Thomas, D. (1979). *Clin. Chem.* **25**, 318
- Boitieux, J.L., Desmet, G., and Thomas, D. (1987). *Enzyme Eng.* **8**, 271
- Boitieux, J.L., Thomas, D., and Desmet, G. (1984). *Anal. Chim. Acta* **163**, 309
- Boivin, P., and Bourdillon, C. (1987). *Stud. Biophys.* **119**, 191
- Borrebaeck, C., Börjeson, J., and Mattiasson, B. (1978). *Clin. Chim. Acta* **86**, 267
- Bourdillon, C., Bourgeois, J.P., and Thomas, D. (1979). *Biotechnol. Bioeng.* **21**, 1877
- Bourdillon, C., Bourgeois, J.P., and Thomas, D. (1980). *J. Chem. Soc.* **102**, 4231
- Bourdillon, C., Thomas, V., and Thomas, D. (1982). *Enzyme Microb. Technol.* **4**, 175
- Boutelle, M., Stanford, C., Fillenz, G., Albery, W.J., and Bartlett, P. (1986). *Neurosci. Lett.* **72**, 283

- Bowers, L.D., and Carr, P.W. (1976). *Clin. Chem.* **22**, 1427
- Bradley, C., and Rechnitz, G.A. (1986). *Anal. Lett.* **19**, 151
- Brahman, J.C., Broeze, R.J., Bowden, D.W., Myles, A., Fulton, T.R., Rising, M., Thurston, J., Cole, F.X., and Vovis, G.F. (1984). *Biotechnology* **4**, 349
- Brooks, C.J.W., and Smith, A.G. (1975). *J. Chromatogr.* **112**, 499
- Brooks, S., Ashby, R., Turner, A.P.F., Calder, M., and Clarke, D.J. (1987/88). *Biosensors* **3**, 45
- Buchholz, K., and Gödelmann, B. (1978). *Biotechnol. Bioeng.* **20**, 1201
- Bülow, L., and Mosbach, K. (1987). *Ann. N.Y. Acad. Sci.* **501**, 44
- Burgmayer, P., and Murray, R. (1982). *J. Am. Chem. Soc.* **104**, 6139
- Burstein, C., Adamowicz, E., Boucherit, K., Rabouille, C., and Romette, J.-L. (1986). *Appl. Biochem. Biotechnol.* **12**, 1
- Bush, D.L., and Rechnitz, G.A. (1987). *Anal. Lett.* **20**, 1781
- Bush, D.L., and Rechnitz, G.A. (1988). *Anal. Lett.* **21**, 1947
- Campanella, L., Cordatore, M., Morabito, R., and Tomassetti, M. (1984). *Abstr. Electrochemical Sensor Symp., Rome*, p. 62
- Campanella, L., Tomassetti, M., and Sammartino, M.-P. (1988). *Analyst* **113**, 994
- Canfield, R.E., and Lu, A.K. (1965). *J. Biol. Chem.* **240**, 1997
- Caras, S., and Janata, J. (1980). *Anal. Chem.* **52**, 1935
- Caras, S., and Janata, J. (1985). *Anal. Chem.* **57**, 1928
- Cardosi, M., and Turner, A.P.F. (1987). In: *Biosensors* (Turner, A.P.F., Karube, I., and Wilson, G.S., Eds.), Oxford University Press, Oxford, p. 257
- Carr, P.W., and Bowers, L.D. (1980). *Immobilized Enzymes in Analytical and Clinical Chemistry*, Wiley, New York
- Cass, A.E.G., Davis, G., Francis, G.D., Hill, H.A.O., Aston, W.J., Higgins, I.J., Plotkin, E.V., Scott, L.D.L., and Turner, A.P.F. (1984). *Anal. Chem.* **56**, 667
- Castner, J.F., and Wingard, L.B. (1984). *Biochemistry* **23**, 2203
- Cenas, N.K., and Kulys, J.J. (1981). *Bioelectrochem. Bioenerg.* **8**, 103
- Cenas, N.K., Rozgaite, M.V., and Kulys, J.J. (1984). *Biotechnol. Bioeng.* **26**, 551
- Chen, A.K., and Liu, C.C. (1977). *Biotechnol. Bioeng.* **19**, 1785
- Chen, A.K., Liu, C.C., and Schiller, J.G. (1979). *Biotechnol. Bioeng.* **21**, 1905
- Clark, L.C. (1965). US Pat. 494 215
- Clark, L.C. (1979). US Pat. 3539 455
- Clark, L.C. (1972). *Biotechnol. Bioeng. Symp.* **3**, 377
- Clark, L.C. (1973). *Am. J. Mental Defic.* **77**, 633
- Clark, L.C. (1977). US Pat. 4040 908
- Clark, L.C. (1979). *Birth Defence* **15**, 37
- Clark, L.C., and Duggan, C.A. (1982). *Diabetes Care* **5**, 174
- Clark, L.C., and Lyons, C. (1962). *Ann. N.Y. Acad. Sci.* **102**, 29
- Clark, L.C., Emory, C., Glueck, C.J., and Campbell, M. (1978). *Enzyme Eng.* **3**, 409
- Clark, L.C., Noyes, L.K., Grooms, T.A., and Gleason, C.A. (1984a). *Clin. Biochem.* **17**, 288
- Clark, L.C., Noyes, L.K., Grooms, T.A., and Moore, M.S. (1984b). *Crit. Care Med.* **12**, 461
- Cleland, V., and Enfors, S.-O. (1984). *Anal. Chem.* **56**, 1880
- Comtat, M., Galy, M., Goulas, P., and Soupe, J. (1988). *Anal. Chim. Acta* **208**, 295
- Corcoran, C.A., and Kobos, R.K. (1983). *Anal. Lett.* **16**, 1291

- Cordonnier, M., Lawny, F., Chapot, D., and Thomas, D. (1975). *Febs Lett.* **59**, 263
- Coulet, P.R. (1987). *GBF Monographs* **10**, 75
- Coulet, P.R., and Blum, L.J. (1983). *Anal. Lett.* **16**, 541
- Cserfalvi, T., and Guilbault, G.G. (1976). *Anal. Chim. Acta* **84**, 259
- Cuyppers, P.A., Hermens, W.T.H., and Hemker, H.C. (1978). *Anal. Biochem.* **84**, 56
- Danielsson, B. (1982). *Appl. Biochem. Biotechnol.* **7**, 127
- Danielsson, B., and Mosbach, K. (1974). *Biochim. Biophys. Acta* **364**, 140
- Danielsson, B., and Mosbach, K. (1988). *Methods Enzymol.* **137D**, 186
- Danielsson, B., Gadd, K., Mattiasson, B., and Mosbach, K. (1976). *Anal. Lett.* **9**, 987
- Danielsson, B., Gadd, K., Mattiasson, B., and Mosbach, K. (1977). *Clin. Chim. Acta* **81**, 163
- Danielsson, B., Lundström, J., Mosbach, K., and Stibler, J. (1979). *Anal. Lett.* **12**, 1189
- Danielsson, B., Mattiasson, B., and Mosbach, K. (1981). *Appl. Biochem. Bioeng.* **3**, 97
- Danielsson, B., Scheller, F., and Schubert, F. (1990) *Enzyme Eng.* **10**, in press.
- Davis, G., Hill, H.A.O., Higgins, I.J., and Turner, A.P.F. (1985). In: *Implantable Sensors for Closed-Loop Prosthetic Systems* (Ko, W.H., Ed.), Future Publishing Co., Mount Kisco, p. 1985
- Davis, P., and Mosbach, K. (1974). *Biochim. Biophys. Acta* **370**, 329
- D'Costa, E.J., Higgins, I.J., and Turner, A.P.F. (1986). *Biosensors* **2**, 71
- De Alwis, W.U., and Wilson, G.S. (1985). *Anal. Chem.* **57**, 2754
- De Alwis, W.U., and Wilson, G.S. (1987). *Anal. Chem.* **59**, 2786
- Decristoforo, G., and Danielsson, B. (1984). *Anal. Chem.* **56**, 263
- Degani, Y., and Heller, A. (1987). *J. Phys. Chem.* **91**, 1285
- Denis, C., Dormois, D., Linossier, M.-T., and Geyssant, A. (1985). *J. Physiol. (Paris)*. **80**, 168
- Dicks, J., Aston, W.J., Davis, G., and Turner, A.P.F. (1986). *Anal. Chim. Acta* **182**, 103
- Dietschy, J.M., Weeks, L.E., and Delente, J.J. (1976). *Clin. Chim. Acta* **73**, 407
- Di Gleria, K., Hill, H.A.O., McNeil, C.J., and Green, M.J. (1986). *Anal. Chem.* **56**, 1203
- Di Paolantonio, C.L., and Rechnitz, G.A. (1982). *Anal. Chim. Acta* **141**, 1
- Di Paolantonio, C.L., and Rechnitz, G.A. (1983). *Anal. Chim. Acta* **148**, 1
- Di Paolantonio, C.L., Arnold, M.A., and Rechnitz, G.A. (1981). *Anal. Chim. Acta* **128**, 121
- Dittmer, H., Pfeiffer, D., and Scheller, F. (1988). Poster, 5th Bucher Symposium, Berlin
- Divies, C. (1975). *Ann. Microbiol. (Paris)*, **126**, 175
- Downs, M.E.A., Kobayashi, S., and Karube, I. (1987). *Anal. Lett.* **20**, 1897
- Doyle, M.J., Halsall, H.B., and Heineman, W.R. (1982). *Anal. Chem.* **54**, 2318
- Doyle, M.J., Halsall, H.B., and Heineman, W.R. (1984). *Anal. Chem.* **56**, 2355
- Dransfeld, I., Hintsche, R., Moritz, W., Pham, M.T., and Hoffmann, W. (1990). *Anal. Lett.* **23**, 437
- Dryhurst, G., Kadish, K., Scheller, F., and Renneberg, R. (1982). *Biological Electrochemistry*, vol. 1, Academic Press, New York
- Durand, P., David, A., and Thomas, D. (1987). *Biochim. Biophys. Acta* **527**, 277
- Durand, P., Nicaud, J., and Mallevialle, J. (1984). *J. Anal. Toxicol.* **8**, 112
- Durliat, H., and Comtat, M. (1978). *J. Electroanal. Chem.* **89**, 221
- Durliat, H., and Comtat, M. (1984). *Anal. Chem.* **56**, 148

- Durliat, H., Comtat, M., and Mahenc, J. (1979). *Anal. Chim. Acta* **106**, 131
- Durst, R.A., and Blubaugh, E.A. (1986). *ACS Symp. Ser.* **309**, 245
- Eggers, H.M., Halsall, H.B., and Heineman, W.R. (1982). *Clin. Chem.* **28**, 1848
- Eldefrawi, M., Sherby, S., Andreou, A., Mansour, N., Arman, Z., Blum, N., and Valdes, J. (1988). *Anal. Lett.* **21**, 1665
- Elving, P.J., Bresnahan, W., Moiroux, J., and Samec, Z. (1982). *Bioelectrochem. Bioenerg.* **9**, 365
- Elwing, H., and Stenberg, M. (1981). *J. Immunol. Methods* **44**, 343
- Endo, J., Tabata, M., Okada, S., and Murachi, T. (1979). *Clin. Chim. Acta* **95**, 411
- Enfors, S.-O. (1981). *Enzyme Microb. Technol.* **3**, 29
- Enfors, S.-O. (1982). *Appl. Biochem. Biotechnol.* **7**, 113
- Enfors, S.-O. (1987). In: *Biosensors* (Turner, A.P.F., Karube, I., and Wilson, G.S., Eds.), Oxford University Press, Oxford, p. 347
- Enfors, S.-O., and Nilsson, H.J. (1979). *Enzyme Microb. Technol.* **1**, 260
- Fatibello-Filho, O., Suleiman, A.A., Guilbault, G.G., and Lubrano, G.J. (1988). *Anal. Chem.* **60**, 2397
- Fawcett, N., Evans, J., Chien, L.-C., and Flowers, N. (1988). *Anal. Lett.* **21**, 1095
- Fife, P.C. (1979). *Mathematical Aspects of Reacting and Diffusing Systems*, Lecture Notes in Biomathematics, vol. 28, Springer, Berlin
- Fiocchi, J.A., and Arnold, M.A. (1984). *Anal. Lett.* **17**, 2091
- Flanagan, M.T., and Carroll, N. (1986). *Biotechnol. Bioeng.* **28**, 1093
- Flanagan, M.T., and Pantell, R.H. (1984). *Electronics Lett.* **20**, 968
- Fogt, E.J., Dodd, L.M., Jennings, E.M., and Clemens, A.H. (1978). *Clin. Chem.* **24**, 1366
- Fonong, T. (1986a). *Anal. Chim. Acta* **184**, 287
- Fonong, T. (1986b). *Anal. Chim. Acta* **186**, 301
- Fonong, T. (1987). *Anal. Lett.* **20**, 783
- Fonong, T., and Rechnitz, G.A. (1984a). *Anal. Chim. Acta* **158**, 357
- Fonong, T., and Rechnitz, G.A. (1984b). *Anal. Chem.* **56**, 2586
- Foulds, N., and Lowe, C.R. (1986). *J. Chem. Soc. Faraday Trans. I* **82**, 1259
- Free, A.H., Adams, E.C., Kercher, M.L., Free, H.M., and Cook, M.H.M. (1956). *Abst. Int. Congr. Clinical Chemistry*, New York, p.235
- Freeman, W., and Seitz, W. (1970). *Anal. Chem.* **50**, 1242
- Fu, H., Anzai, J.-J., Osa, T., and Matsuo, T. (1988). *Chem. Pharm. Bull.* **36**, 1190
- Fuh, M.-R.S., Burgess, L.W., and Christian, G.D. (1988). *Anal. Chem.* **60**, 433
- Gebauer, C.R., and Rechnitz, G.A. (1982). *Anal. Biochem.* **124**, 338
- Geppert, G., and Asperger, L. (1987). *Bioelectrochem. Bioenerg.* **17**, 399
- Geyssant, A., Dormois, D., Barthelemy, J.C., and Lacour, J.R. (1985). *Scand. J. Clin. Lab. Invest.* **45**, 145
- Giaever, I., Keese, C.R., and Ryves, R.I. (1984). *Clin. Chem.* **30**, 880
- Glad, C., Sjödin, K., and Mattiasson, B. (1986). *Biosensors* **2**, 89
- Goldfinch, M.J., and Lowe, C.R. (1980). *Anal. Biochem.* **109**, 216
- Goldfinch, M.J., and Lowe, C.R. (1984). *Anal. Biochem.* **138**, 430
- Gondo, S., Osaki, T., and Morishita, M. (1981). *J. Mol. Catal.* **12**, 365
- Gorton, L., and Ögren, L. (1981). *Anal. Chim. Acta* **130**, 45
- Gorton, L., Scheller, F., and Johansson, G. (1985). *Studia Biophys.* **109**, 199
- Gotoh, M., Tamiya, E., Momoi, M., Kagawa, Y., and Karube, I. (1987). *Anal. Lett.* **20**, 857

- Gough, D.A., Lucisano, J.Y., and Tse, P.S.H. (1985). *Anal. Chem.* **57**, 2351
- Green, M.J., Hill, H.A.O., Tew, D.G., and Walton, N.J. (1984). *Febs Lett.* **170**, 69
- Grobler, S.R., and Rechnitz, G.A. (1980). *Talanta* **27**, 283
- Gruss, R. (1989). Dissertation, Academy of Sciences of the GDR, Berlin
- Gruss, R., and Scheller, F. (1987). *Z. Med. Lab.-Diagn.* **28**, 333
- Guanasekaran, R., and Mottola, H.A. (1985). *Anal. Chem.* **57**, 1005
- Guilbault, G.G. (1976). *Handbook of Enzymatic Methods of Analysis*, Marcel Dekker, New York, p. 490
- Guilbault, G.G. (1980). *Ion Select. Electrode Rev.* **2**, 3
- Guilbault, G.G., and Coulet, P.R. (1983). *Anal. Chim. Acta* **152**, 223
- Guilbault, G.G., and Hrabankóva, E. (1970). *Anal. Chim. Acta* **52**, 287
- Guilbault, G.G., and Hrabankóva, E. (1971). *Anal. Chim. Acta* **56**, 285
- Guilbault, G.G., and Lubrano, G. (1973). *Anal. Chim. Acta* **64**, 439
- Guilbault, G.G., and Lubrano, G. (1974). *Anal. Chim. Acta* **69**, 183
- Guilbault, G.G., and Montalvo, J. (1969). *J. Am. Chem. Soc.* **91**, 2164
- Guilbault, G.G., and Nagy, G. (1973). *Anal. Chem.* **45**, 417
- Guilbault, G.G., and Nanjo, M. (1975a). *Anal. Chim. Acta* **75**, 169
- Guilbault, G.G., and Nanjo, M. (1975b). *Anal. Chim. Acta* **78**, 69
- Guilbault, G.G., and Ngeh-Ngwainbi, J. (1987). *GBF Monographs* **10**, 187
- Guilbault, G.G., and Starklov, W. (1975). *Anal. Chim. Acta* **76**, 237
- Guilbault, G.G., and Tarp, M. (1974). *Anal. Chim. Acta* **73**, 355
- Guilbault, G.G., Smith, R., and Montalvo, J. (1969). *Anal. Chem.* **41**, 600
- Guilbault, G.G., Danielsson, B., Mandenius, C.F., and Mosbach, K. (1983). *Anal. Chem.* **55**, 1582
- Guilbault, G.G., Czarnecki, J., and Rahni, M.A.N. (1985). *Anal. Chem.* **57**, 2110
- Guilbault, G.G., Lubrano, G.J., Kauffmann, J.-M., and Patriarche, G.J. (1988). *Anal. Chim. Acta* **206**, 369
- Gyss, C., and Bourdillon, C. (1987). *Anal. Chem.* **59**, 2350
- Hafeman, D.G., Parce, J.W., and McConnell, H.M. (1988). *Science* **240**, 1182
- Haga, M., Itagaki, H., and Sugawara, S. (1980). *Biochem. Biophys. Res. Commun.* **95**, 187
- Haga, M., Ikuta, M., Kato, Y., and Suzuki, Y. (1984). *Chem. Lett.* 1313
- Hall, E. (1986). *Enzyme Microb. Technol.* **8**, 651
- Hall, E., Best, D., and Turner, A.P.F. (1988). *Enzyme Microb. Technol.* **10**, 543
- Hamann, H. (1988). Dissertation, Academy of Sciences of the GDR, Berlin
- Hamann, H., Kühn, M., Böttcher, N., and Scheller, F. (1986). *J. Electroanal. Chem.* **209**, 69
- Hanazato, Y., and Shiono, S. (1983). *Proc. Int. Meeting Chemical Sensors*, Fukuoka, Elsevier, Amsterdam, p. 513
- Hanazato, Y., Nakako, N., Maeda, M., and Shiono, S. (1986). *Proc. 2nd Int. Meeting Chemical Sensors*, Bordeaux, p. 576
- Hanazato, Y., Inatomi, K.-I., Nakako, N., Shiono, S., and Maeda, M. (1988). *Anal. Chim. Acta* **213**, 49
- Hanke, G. (1989). Dissertation, Academy of Sciences of the GDR, Berlin
- Hanke, G., Scheller, F., and Yersin, A. (1987). *Zentralbl. Pharm.* **126**, 445
- Hanus, F., Carter, K., and Evans, H. (1980). *Methods Enzymol.* **69C**, 731
- Harris, C., and Kell, D.B. (1985). *Biosensors* **1**, 17

- Harrison, D., Turner, R.F.B., and Baltes, H.P. (1988). *Anal. Chem.* **60**, 2002
- Hassan, S.S.M., and Rechnitz, G.A. (1981). *Anal. Chem.* **53**, 512
- Hauptmann, B. (1985). M.S. Thesis, Technical University Magdeburg, Magdeburg
- Hayashi, K., Uji, K.Y., Toko, K., Ozaki, N., Yoshida, T., Iiyame, S., and Nakashima, N. (1989). *Sensors Actuat.* **16**, 25
- Heineman, W.R., and Halsall, H.B. (1985). *Anal. Chem.* **57**, 1321A
- Heller, A., and Degani, Y. (1987). *J. Electrochem. Soc. Rev. News* **134**, 494C
- Higgins, I.J., and Lowe, C.R. (1987). *Phil. Trans. R. Soc. London* **316B**, 3
- Higgins, I.J., Bannister, J.V., and Turner, A.P.F. (1987). *GBF Monographs* **10**, 23
- Hikuma, M., Kubo, T., Yasuda, T., Karube, I., and Suzuki, S. (1979a). *Biotechnol. Bioeng.* **21**, 1845
- Hikuma, M., Kubo, T., Yasuda, T., Karube, I., and Suzuki, S. (1979b). *Anal. Chim. Acta* **109**, 33
- Hikuma, M., Suzuki, H., Yasuda, T., Karube, I., and Suzuki, S. (1979c). *Eur. J. Appl. Microbiol. Biotechnol.* **8**, 289
- Hikuma, M., Obana, H., and Yasuda, T. (1980a). *Enzyme Microb. Technol.* **2**, 234
- Hikuma, M., Kubo, T., Yasuda, T., Karube, I., and Suzuki, S. (1980b). *Anal. Chem.* **52**, 1020
- Hikuma, M., Obana, H., Yasuda, T., Karube, I., and Suzuki, S. (1980c). *Anal. Chim. Acta* **116**, 61
- Hikuma, M., Suzuki, H., Yasuda, T., Karube, I., and Suzuki, S. (1980d). *Eur. J. Appl. Microbiol. Biotechnol.* **9**, 305
- Hill, H.A.O., Walton, N.J., and Higgins, I.J. (1981). **126**, 282
- Hintsche, R., and Scheller, F. (1987). *Studia Biophys.* **119**, 179
- Hintsche, R., Neumann, G., Dransfeld, I., Kampfrath, G., and Scheller, F. (1990). *Biosensors* **5**, in press
- Ho, M.H., and Wu, T.G. (1985). *ISA Trans.* **24**, 61
- Ho, M.Y.K., and Rechnitz, G.A. (1985). *Biotechnol. Bioeng.* **27**, 1634
- Ho, M.Y.K., and Rechnitz, G.A. (1987). *Anal. Chem.* **59**, 536
- Hofmann, E. (1984). *Enzyme und Bioenergetik, Dynamische Biochemie*, vol. 2, Akademie-Verlag Berlin, Berlin, p. 13
- Honold, F., and Cammann, K. (1987). *GBF Monographs* **10**, 285
- Hopkins, T.R. (1985). *Int. Biotechnol. Lab.* **3**, 20
- Huang, H.S., Kuan, J.W., and Guilbault, G.G. (1977). *Clin. Chem.* **23**, 671
- Huck, H., Schelter-Graf, A., and Schmidt, H.-L. (1984). *Bioelectrochem. Bioenerg.* **13**, 199
- Ianiello, R.M., and Yacynych, A.M. (1981). *Anal. Chem.* **53**, 2090
- Ianiello, R.M., and Yacynych, A.M. (1983). *Anal. Chim. Acta* **146**, 249
- Ianiello, R.M., Lindsay, T.J., and Yacynych, A.M. (1982a). *Anal. Chem.* **54**, 1980
- Ianiello, R.M., Lindsay, T.J., and Yacynych, A.M. (1982b). *Anal. Chem.* **54**, 1098
- Iida, T., Kurube, T., Hisatomi, M., and Mitamura, T. (1986). *Proc. 2nd Int. Meeting Chemical Sensors, Bordeaux*, p. 592
- Ikariyama, Y., Furuki, M., and Aizawa, M. (1983). *Proc. Int. Meeting Chemical Sensors, Fukuoka, Elsevier, Amsterdam*, p. 693
- Ikariyama, Y., Yamauchi, S., Yukiashi, T., and Ushida, H. (1987). *Anal. Lett.* **20**, 1407
- Ikariyama, Y., Shimada, N., and Yamauchi, S. (1988). *Anal. Lett.* **21**, 953
- Ikeda, T., Katasho, J., Kamei, M., and Senda, M. (1984). *Agric. Biol. Chem.* **48**, 1969

- Ikeda, T., Hamada, H., Miki, K., and Senda, M. (1985). *Agric. Biol. Chem.* **49**, 541
- Ikeda, T., Miki, K., Fushimi, F., and Senda, M. (1987). *Agric. Biol. Chem.* **51**, 747
- Ikeda, T., Miki, K., Fushimi, F., and Senda, M. (1988a). *Agric. Biol. Chem.* **52**, 1557
- Ikeda, T., Fushimi, F., Miki, K., and Senda, M. (1988b). *Agric. Biol. Chem.* **52**, 2655
- Ishikara, K., Muramoto, N., Fujii, H., and Shinohara, I. (1985). *J. Polymer Sci. Polymer Lett.* **23**, 531
- Ishimori, Y., Yasuda, T., Tsumita, T., Notsuki, M., Koyama, M., and Tadakuma, T. (1984). *J. Immunol. Methods* **75**, 351
- IUPAC and IUB (1973). *Enzyme Nomenclature, Recommendations*, Elsevier, Amsterdam, chapter 4
- Janata, J. (1975). *J. Am. Chem. Soc.* **97**, 2914
- Janata, J. (1985). *Anal. Chem.* **57**, 1924
- Janata, J., and Huber, R.J. (1980). In: *Ion-Selective Electrodes in Analytical Chemistry* (Freiser, H., Ed.), vol. 2, Plenum Press, New York, p. 107
- Janata, J., and Moss, S. (1976). *Biomed. Eng.* **11**, 141
- Jänchen, M., Pfeiffer, D., Scheller, O., and Scheller, F. (1980). *Z. Med. Lab.-Diagn.* **21**, 325
- Jänchen, M., Walzel, G., Neef, B., Wolf, B., Scheller, F., Kühn, M., and Pfeiffer, D. (1983). *Biomed. Biochim. Acta* **42**, 1055
- Jasaitis, J.J., Razumas, V.J., and Kulys, J.J. (1983). *Anal. Chim. Acta* **152**, 271
- Jehring, H., Huyen, N.V., Gian, T.X., Horn, E., and Herchi, C. (1979). *J. Electroanal. Chem.* **100**, 13
- Jensen, M.A., and Rechnitz, G.A. (1978). *Anal. Chim. Acta* **101**, 125
- Jensen, M.A., and Rechnitz, G.A. (1979). *J. Membrane Sci.* **5**, 117
- Johnson, J.M., Halsall, H.B., and Heineman, W.R. (1982). *Anal. Chem.* **54**, 1394
- Johnson, J.M., Halsall, H.B., and Heineman, W.R. (1985). *Biochemistry* **24**, 1579
- Jönsson, G., and Gorton, L. (1985). *Biosensors* **1**, 355
- Joseph, J. (1984). *Microchim. Acta* **II**, 473
- Kamke, E. (1956). *Differentialgleichungen*, vol. 1, Akademische Verlagsgesellschaft, Leipzig
- Kampfrath, G., Hoffmann, W., and Hintsche, R. (1989). *Anal. Lett.* **22**, 2423
- Karube, I. (1986). *Sci. Technol. Japan*, July/Sept., 32
- Karube, I. (1988). *Kihan Kagaku Soetsu* **1**, 102
- Karube, I., and Morizumi, T. (1988). *Methods Enzymol.* **137 D**, 255
- Karube, I., and Tamiya, E. (1986). *Proc. 2nd Int. Meeting Chemical Sensors*, Bordeaux, p. 588
- Karube, I., Matsunaga, T., and Suzuki, S. (1977a). *J. Solid-Phase Biochem.* **2**, 97
- Karube, I., Matsunaga, T., Mitsuda, S., and Suzuki, S. (1977b). *Biotechnol. Bioeng.* **19**, 1535
- Karube, I., Matsunaga, T., Nakahara, T., and Suzuki, S. (1977c). *Biotechnol. Bioeng.* **19**, 1727
- Karube, I., Matsunaga, T., and Suzuki, S. (1979a). *Anal. Chim. Acta* **109**, 39
- Karube, I., Mitsuda, S., and Suzuki, S. (1979b). *Eur. J. Appl. Microbiol. Biotechnol.* **7**, 343
- Karube, I., Hara, K., Satoh, I., and Suzuki, S. (1979c). *Anal. Chim. Acta* **106**, 243
- Karube, I., Suzuki, S., Okada, T., and Hikuma, M. (1980a). *Biochimie* **62**, 567

- Karube, I., Satoh, I., Araki, Y., Suzuki, S., and Yamada, H. (1980b). *Enzyme Microb. Technol.* **2**, 117
- Karube, I., Okada, T., and Suzuki, S. (1981a). *Anal. Chem.* **53**, 1952
- Karube, I., Matsunaga, T., Nakahara, T., and Suzuki, S. (1981b). *Anal. Chem.* **53**, 1024
- Karube, I., Okada, T., and Suzuki, S. (1982a). *Anal. Chim. Acta* **135**, 61
- Karube, I., Hara, K., Matsuoka, H., and Suzuki, S. (1982b). *Anal. Chim. Acta* **139**, 127
- Karube, I., Matsunaga, T., Nakahara, T., and Suzuki, S. (1982c). *Anal. Chem.* **54**, 1725
- Karube, I., Okada, T., Suzuki, S., Suzuki, H., Hikuma, M., and Yasuda, T. (1982d). *Eur. J. Appl. Microbiol. Biotechnol.* **15**, 127
- Karube, I., Sogabe, S., Matsunaga, T., and Suzuki, S. (1983). *Eur. J. Appl. Microbiol. Biotechnol.* **17**, 216
- Karube, I., Tamiga, E., Dicks, J., and Gotoh, M. (1986). *Anal. Chim. Acta* **185**, 195
- Katsu, T., Kanamitsu, M., and Hirota, T. (1986). *Chem. Pharm. Bull.* **34**, 3968
- Kawashima, T., and Rechnitz, G.A. (1976). *Anal. Chim. Acta* **83**, 9
- Kawashima, T., Arima, A., Hatakeyama, N., Taminaga, N., and Ando, H. (1980). *J. Chem. Soc. Japan* **10**, 1542
- Kawashima, T., Tomida, K., Tominaga, N., Kobayashi, T., and Onishi, H. (1984). *Chem. Lett.* 653
- Keating, M.Y., and Rechnitz, G.A. (1983). *Analyst* **108**, 766
- Keating, M.Y., and Rechnitz, G.A. (1984). *Anal. Chem.* **56**, 801
- Kessler, M., Höper, J., Volkholz, H.J., Sailer, D., and Demling, L. (1984). *Hepato-Gastroenterol.* **31**, 285
- Kiang, C.-H., Kuan, S., and Guilbault, G.G. (1978). *Anal. Chem.* **50**, 1319
- Kiesewetter, M., Möricke, R., Wernstedt, J., and Lembke, K. (1985). Lecture at the 30th Int. Sci. Kolloquium, Ilmenau
- Kihara, K., Yasukawa, E., Hayashi, M., and Hirose, S. (1984a). *Anal. Chim. Acta* **159**, 81
- Kihara, K., Yasukawa, E., Hayashi, M., and Hirose, S. (1984b). *Anal. Chem.* **56**, 1376
- Kimura, J., Kuriyama, T., and Kawana, Y. (1985). *Proc. 2nd Int. Conf. Solid-State Sensors and Actuators*, Philadelphia, p. 152
- Kimura, J., Kawana, Y., and Kuriyama, T. (1989). *Biosensors* **4**, 41
- Kirstein, D., Scheller, F., and Mohr, P. (1980). *Acta Biotechnol.* **0**, 65
- Kirstein, D., Kirstein, L., and Scheller, F. (1985a). *Biosensors* **1**, 117
- Kirstein, D., Scheller, F., Olsson, B., and Johansson, G. (1985b). *Anal. Chim. Acta* **171**, 345
- Kirstein, D., Danielsson, B., Scheller, F., and Mosbach, K. (1987). *Proc. 4th Eur. Congr. Biotechnology*, Elsevier, Amsterdam, p. 215
- Kirstein, D., Danielsson, B., Scheller, F., and Mosbach, K. (1989). *Biosensors* **4**, 231
- Kirstein, L. (1987). Dissertation, Humboldt University, Berlin
- Kitagawa, Y., Tamiya, E., and Karube, I. (1987). *Anal. Lett.* **20**, 81
- Kjellén, K.G., and Neujahr, H.Y. (1980). *Biotechnol. Bioeng.* **22**, 299
- Klopper, W.J., Angelino, S.A.G.F., Knol, W., and Minekus, M. (1987). *Proc. 21st European Brewery Convention Congress*, Madrid, Oxford Information Printing, Oxford, p. 87
- Kobayashi, T., and Laidler, K. (1974). *Biotechnol. Bioeng.* **16**, 77

- Kobayashi, T., Saga, K., Shimizu, S., and Goto, T. (1981). *Agric. Biol. Chem.* **45**, 1403
- Kobos, R.K. (1986). *Anal. Lett.* **19**, 353
- Kobos, R.K., and Pyon, H.Y. (1981). *Biotechnol. Bioeng.* **23**, 627
- Kobos, R.K., and Ramsay, T. (1980). *Anal. Chim. Acta* **121**, 111
- Kobos, R.K., and Rechnitz, G.A. (1977). *Anal. Lett.* **10**, 751
- Kobos, R.K., Rice, D.J., and Flournoy, D.S. (1979). *Anal. Chem.* **51**, 1122
- Kobos, R.K., Eveleigh, J.W., Stapler, M.L., Haley, B.J., and Papa, S.L. (1988). *Anal. Chem.* **60**, 1996
- Kooyman, R.P.H., Kolkman, H., and Greve, J. (1987). *GBF Monographs* **10**, 295
- Koshy, K., Bennetto, H.P., Delaney, G.M., MacLevel, A.J., Mason, J.R., Sterling, J.L., and Thurston, C.F. (1988). *Anal. Lett.* **21**, 2177
- Kotowski, J., Janas, T., and TiTien, H. (1988). *Bioelectrochem. Bioenerg.* **19**, 277
- Koyama, M., Satoh, Y., Aizawa, M., and Suzuki, S. (1980). *Anal. Chim. Acta* **116**, 307
- Kress-Rogers, E. (1985). *Food Proc.*, Sept., 37
- Kronick, M.N., and Little, W.A. (1973). *J. Immunol. Methods* **8**, 235
- Krull, U.J., and Thompson, M. (1985). *Trends Anal. Chem.* **4**, 90
- Kuan, J.W., and Guilbault, G.G. (1977). *Clin. Chem.* **23**, 1058
- Kuan, J.W., Kuan S.S., and Guilbault, G.G. (1978). *Anal. Chim. Acta* **100**, 220
- Kubo, I., Karube, I., and Suzuki, S. (1983a). *Anal. Chim. Acta* **151**, 371
- Kubo, I., Osawa, M., Karube, I., Matsuoka, M., and Suzuki, S. (1983b). *Proc. Int. Meeting Chemical Sensors*, Fukuoka, Elsevier, Amsterdam, p. 660
- Kulys, J.J. (1981). *Anal. Lett.* **14**, 377
- Kulys, J.J. (1986). *Biosensors* **2**, 1
- Kulys, J.J. and Cenas, N. (1983). *Biochim. Biophys. Acta* **744**, 57
- Kulys, J.J. and Kadziauskiene, K.-V. (1978). *Dokl. Akad. Nauk* **239**, 636
- Kulys, J.J. and Kadziauskiene, K.-V. (1980). *Biotechnol. Bioeng.* **22**, 221
- Kulys, J.J. and Malinauskas, A. (1979a). *Zh. Anal. Khim.* **24**, 876
- Kulys, J.J. and Malinauskas, A. (1979b). *Biotechnol. Bioeng.* **21**, 876
- Kulys, J.J. and Svirmickas, G.J.S. (1980a). *Febs Lett.* **114**, 7
- Kulys, J.J. and Svirmickas, G.J.S. (1980b). *Anal. Chim. Acta* **117**, 115
- Kulys, J.J. and Vidziunaite, R.A. (1983). *Anal. Lett.* **16**, 197
- Kulys, J.J., Ralis, E.V., and Penkova, R.S. (1979). *Priklad. Biokhim. Mikrobiol.* **15**, 282
- Kulys, J.J., Gureviciene, V.V., and Laurinavicius, V.A. (1980). *Antibiotiki* **25**, 655
- Kulys, J.J., Cenas, N.K., Svirmickas, G.J.S., and Svirmickiene, V.P. (1982). *Anal. Chim. Acta* **138**, 19
- Kulys, J.J., Laurinavicius, V.A., Pesliakiene, M.W., and Gureviciene, V.A. (1983). *Anal. Chim. Acta* **148**, 13
- Kulys, J.J., Pesliakiene, M.W., Laurinavicius, V.V., Tatikyan, S., and Simonyan, A.L. (1985). *Zh. Anal. Khim.* **11**, 2077
- Kulys, J.J., Sorochinskii, V.V., and Vidziunaite, R.A. (1986a). *Biosensors* **2**, 135
- Kulys, J.J., Gureviciene, V.V., and Laurinavicius, V.A. (1986b). *Biosensors* **2**, 35
- Kumar, A., and Christian, G.D. (1977). *Clin. Chim. Acta* **74**, 101
- Kuriyama, S., and Rechnitz, G.A. (1981). *Anal. Chim. Acta* **131**, 91
- Kuriyama, S., Arnold, M.A., and Rechnitz, G.A. (1983). *J. Membrane Sci.* **12**, 269
- Kuriyama, T., Nakamoto, S., Kawame, Y., and Kimura, J. (1986). *Proc. 2nd Int. Meeting Chemical Sensors*, Bordeaux, p. 568

- Lang, F., Gstrein, E., Geibel, J., Rehwald, W., Völkl, H., and Oberleitner, H. (1983). *Bioelectrochem. Bioenerg.* **11**, 365
- Lasia, A. (1983). *J. Electroanal. Chem.* **146**, 397
- Laval, J.-M., Bourdillon, C., and Moiroux, J. (1984). *J. Am. Chem. Soc.* **106**, 4701
- Layne, E.C., Schultz, R.D., Thomas, L.J., Slama, G., Sayler, D.F., and Bessman, S.P. (1976). *Diabetes* **25**, 81
- Leypoldt, J.K., and Gough, D.A. (1984). *Anal. Chem.* **56**, 2896
- Libeer, J.C. (1985). *J. Clin. Chem. Clin. Biochem.* **23**, 645
- Liedberg, B., Nylander, C., and Lundström, I. (1983). *Sensors Actuat.* **4**, 299
- Lindberg, A. (1983). *Anal. Chim. Acta* **152**, 113
- Lindh, M., Lindgren, K., Carlström, A., and Masson, P. (1982). *Clin. Chem.* **28**, 726
- Litschko, E. (1988). Poster, 5th Bucher Symposium, Berlin
- Liu, C.C., Wingard, L.B., Wolfson, S.K., Yao, S.J., Drash, A.L., and Schiller, J.G. (1979). *J. Electroanal. Chem.* **104**, 19
- Liu, C.C., Weaver, J.P., and Chen, A. (1981). *Bioelectrochem. Bioenerg.* **8**, 379
- Liu, C.C., Fryburg, M., and Chen, A. (1982). *Bioelectrochem. Bioenerg.* **9**, 103
- Lloyd, D., James, K., Williams, J., and Williams, N. (1981). *Anal. Biochem.* **116**, 17
- Lobel, E., and Rishpon, J. (1981). *Anal. Chem.* **53**, 51
- Lowe, C.R. (1986). Lecture at the Discussion Meeting "Biosensors" of the Royal Society, London
- Lowe, C.R., and Goldfinch, M.J. (1983). *Biochem. Soc. Trans.* **11**, 446
- Lowe, C.R., and Goldfinch, M.J. (1988). *Methods Enzymol.* **137 D**, 338
- Lowe, C.R., Goldfinch, M.J., and Lias, R.J. (1983). *Biotech 83*, Online Publications, Northwood, p. 633
- Lundström, I. (1978). *Phys. Scripta* **18**, 424
- Lundström, I., Spetz, A., and Winquist, F. (1987). *Phil. Trans. R. Soc. London* **316B**, 47
- Lutter, J. (1988). personal communication
- Lynn, K.R., Chuaqui, C.A., and Clevette-Radford, N.A. (1982). *Bioorgan. Chem.* **11**, 19
- Ma, Y.L., and Rechnitz, G.A. (1985). *Anal. Lett.* **18**, 1635
- Macholán, L. (1979). *Coll. Czech. Chem. Commun.* **44**, 3033
- Macholán, L., and Chmelikova, B. (1986). *Anal. Chim. Acta* **185**, 187
- Macholán, L., and Jilek, M. (1984). *Coll. Czech. Chem. Commun.* **49**, 752
- Macholán, L., and Jilkova, D. (1983). *Coll. Czech. Chem. Commun.* **48**, 672
- Macholán, L., and Konecna, M. (1983). *Coll. Czech. Chem. Commun.* **48**, 798
- Macholán, L., and Schanel, L. (1984). *Biologia* **39**, 1191
- Macholán, L., Londyn, P., and Fischer, J. (1981) *Coll. Czech. Chem. Commun.* **46**, 2871
- Malinauskas, A.A., and Kulys, J.J. (1978). *Anal. Chim. Acta* **98**, 31
- Malinauskas, A.A., and Kulys, J.J. (1979). *Biotechnol. Bioeng.* **21**, 513
- Malovik, V., Yaropolov, A., and Varfolomeev, S.D. (1983). *Coll. Czech. Chem. Commun.* **49**, 1390
- Malpiece, Y., Sharan, M., Barbotin, J.-N., Personne, P., and Thomas, D. (1981). *J. Biol. Chem.* **255**, 6883
- Mandenius, C.F., Danielsson, B., and Mattiasson, B. (1981). *Biotechnol. Lett.* **3**, 629
- Mandenius, C.F., Welin, S., Danielsson, B., Lundström, I., and Mosbach, K. (1984). *Anal. Biochem.* **137**, 106

- Mandenius, C.F., Mosbach, K., Welin, S., and Lundström, I. (1986). *Anal. Biochem.* **157**, 283
- Mansson, M.O., Siegbahn, N., and Mosbach, K. (1983). *Proc. Natl. Acad. Sci. USA* **80**, 1487
- Mascini, M. (1987). *GBF Monographs* **10**, 87
- Mascini, M., and Mazzei, F. (1986). *Proc. 2nd Int. Meeting Chemical Sensors, Bordeaux*, p. 611
- Mascini, M., and Memoli, A. (1986). *Anal. Chim. Acta* **182**, 113
- Mascini, M., and Moscone, D. (1986). *Anal. Chim. Acta* **179**, 439
- Mascini, M., and Palleschi, G. (1983a). *Anal. Chim. Acta* **145**, 213
- Mascini, M., and Palleschi, G. (1983b). *Anal. Lett.* **16**, 1053
- Mascini, M., Iannello, M., and Palleschi, G. (1982). *Anal. Chim. Acta* **138**, 65
- Mascini, M., Iannello, M., and Palleschi, G. (1983). *Anal. Chim. Acta* **146**, 135
- Mascini, M., Moscone, D., and Palleschi, G. (1984). *Anal. Chim. Acta* **157**, 45
- Mascini, M., Fortunati, S., Moscone, D., and Palleschi, G. (1985a). *Anal. Chim. Acta* **171**, 175
- Mascini, M., Fortunati, S., Moscone, D., Calabrese, G., Massi-Benedetti, M., and Fabietti, P. (1985b). *Clin. Chem.* **31**, 451
- Mascini, M., Moscone, D., and Palleschi, G. (1986). *Proc. 2nd Int. Meeting Chemical Sensors, Bordeaux*, p. 607
- Mascini, M., Mazzei, F., Moscone, D., Calabrese, G., and Massi-Benedetti, M. (1987). *Clin. Chem.* **33**, 591
- Mason, M. (1983a). *J. Assoc. Off. Anal. Chem.* **66**, 981
- Mason, M. (1983b). *J. Am. Soc. Brewing Chem.* **40**, 78
- Mason, M. (1987). *Lecture at Biotec 87, Düsseldorf*
- Mathot, C., D'Alessandro, P.A., Scher, S., and Rothen, A. (1967). *Am. J. Trop. Med. Hyg.* **16**, 443
- Matsumoto, K., Seiyo, H., Watanabe, T., Karube, I., Satoh, I., and Suzuki, S. (1979). *Anal. Chim. Acta* **105**, 429
- Matsumoto, K., Yamada, K., and Osajima, Y. (1981). *Anal. Chem.* **53**, 1974
- Matsumoto, K., Hamada, O., Ukeda, H., and Osajima, Y. (1985). *Agric. Biol. Chem.* **49**, 2132
- Matsumoto, K., Kamikado, H., Matsubara, H., and Osajima, Y. (1988). *Anal. Chem.* **60**, 147
- Matsunaga, T., and Nakajima, T. (1985). *Appl. Environ. Microbiol.* **50**, 238
- Matsunaga, T., and Namba, Y. (1984a). *Anal. Chim. Acta* **156**, 404
- Matsunaga, T., and Namba, Y. (1984b). *Anal. Chem.* **56**, 798
- Matsunaga, T., Karube, I., and Suzuki, S. (1978). *Anal. Chim. Acta* **99**, 233
- Matsunaga, T., Karube, I., and Suzuki, S. (1980a). *Eur. J. Appl. Microbiol. Biotechnol.* **10**, 125
- Matsunaga, T., Karube, I., and Suzuki, S. (1980b). *Eur. J. Appl. Microbiol. Biotechnol.* **10**, 235
- Matsunaga, T., Karube, I., Nakahara, T., and Suzuki, S. (1981). *Eur. J. Appl. Microbiol. Biotechnol.* **12**, 97
- Matsunaga, T., Suzuki, S., and Tomoda, R. (1984a). *Enzyme Microb. Technol.* **6**, 355
- Matsunaga, T., Tomoda, R., and Matsuda, H. (1984b). *Appl. Microbiol. Biotechnol.* **19**, 404
- Matsuoka, H., and Homma, T. (1989). *Bioelectrochem. Bioenerg.* **21**, 343

- Mattiasson, B. (1977). *Febs Lett.* **77**, 107
- Mattiasson, B. (1984). *Trends Anal. Chem.* **3**, 245
- Mattiasson, B., and Danielsson, B. (1982). *Carbohydr. Res.* **102**, 273
- Mattiasson, B., and Nilsson, H. (1977). *Febs Lett.* **78**, 251
- Mattiasson, B., Danielsson, B., and Mosbach, K. (1976). *Anal. Lett.* **9**, 217
- Mattiasson, B., Nilsson, H., and Olsson, B. (1979). *J. Appl. Biochem.* **1**, 377
- Mattiasson, B., Larsson, P.-O., Lindahl, L., and Sahlin, P. (1982). *Enzyme Microb. Technol.* **4**, 153
- Matuszewski, W., and Trojanowicz, M. (1988). *Analyst* **113**, 735
- McCann, J. (1987). *World Biotech Rep.* **1**, pt. 2, 41
- McKean, B.D., and Gough, D.A. (1988). *IEEE Trans. Biomed. Eng.* **35**, 526
- Merten, O.W. (1988). *Animal Cell Biotechnol.* **3**, 76
- Meyerhoff, M.E., and Rechnitz, G.A. (1976). *Anal. Chim. Acta* **85**, 277
- Meyerhoff, M.E., and Rechnitz, G.A. (1979). *Anal. Biochem.* **95**, 483
- Miki, K., Ikeda, T., and Senda, M. (1985). *Rev. Polarogr. Japan* **31**, 53
- Mindner, K., Flemming, C., and Langhammer, G. (1978). *Z. Med. Lab.-Diagn.* **19**, 222
- Mindt, W., Racine, P., and Schlöpfer, P. (1971). *Swiss Pat.* 13 211
- Mindt, W., Racine, P., and Schlöpfer, P. (1973). *Ber. Bunsenges. Phys. Chem.* **47**, 804
- Miner, D.J., Rice, J.R., Riggins, R.M., and Kissinger, P.T. (1981). *Anal. Chem.* **53**, 2258
- Miyahara, Y., Moriizumi, T., and Ichimura, K. (1985). *Sensors Actuat.* **7**, 1
- Miyahara, Y., Matsu, F., Shiokawa, S., Moriizumi, T., Matsuoka, H., Karube, I., and Suzuki, S. (1983). *Proc. Int. Meeting Chemical Sensors, Fukuoka, Elsevier, Amsterdam*, p. 21
- Mizutani, F. (1982). *Jap. Pat.* 006 961
- Mizutani, F., and Tsuda, K. (1982). *Anal. Chim. Acta* **134**, 359
- Mizutani, F., Tsuda, K., Karube, I., Suzuki, S., and Matsumoto, K. (1980). *Anal. Chim. Acta* **118**, 65
- Mizutani, F., Sasaki, K., and Shimura, Y. (1983). *Anal. Chem.* **55**, 35
- Mizutani, F., Yamanaka, T., Tanabe, Y., and Tsuda, K. (1985). *Anal. Chim. Acta* **177**, 153
- Mohr, P., Scheller, F., Renneberg, R., Kühn, M., Pommerening, K., Schubert, F., and Scheler, W. (1984). In: *Cytochrome P-450* (Ruckpaul, K., and Rein, H., Eds.). Akademie-Verlag Berlin, p. 370
- Morizuma, T., Takatsu, L., and Ono, K. (1986). *Proc. 2nd Int. Meeting Chemical Sensors, Bordeaux*, p. 641
- Moriizumi, T. (1988). *New Technol. Japan* **8**, 29
- Moriizumi, T., and Onoue, Y. (1986). *Jap. Pat.* 86/51 899
- Mosbach, K. (1977). *US Pat.* 4021 307
- Mosbach, K., Blaedel, W.J., Laval, J.-M., Bourdillon, C., and Moiroux, J. (1984). *J. Am. Chem. Soc.* **106**, 4701
- Mottola, H.A. (1983). *Anal. Chim. Acta* **145**, 27
- Muehlbauer, M.J., Guilbeau, E.J., and Towi, B.C. (1989). *Anal. Chem.* **61**, 77
- Mueller, P., Rudin, D.O., TiTien, H., and Wescott, W.C. (1962). *Nature* **194**, 979
- Mullen, W.H. (1986). *Anal. Chim. Acta* **183**, 59
- Mullen, W.H., Churchhouse, S.J., and Vadgama, P.M. (1985). *Analyst* **110**, 952
- Mullen, W.H., Churchhouse, S.J., Keedy, F.H., and Vadgama, P.M. (1986). *Clin. Chim. Acta* **157**, 191

- Müller, A., Abel, P., and Fischer, U. (1986). *Biomed. Biochim. Acta* **45**, 769
- Murakami, T., Takamoto, S., Kimura, I., Kuriyama, T., and Karube, I. (1986). *Anal. Lett.* **19**, 1973
- Muramatsu, H., Kajiwarra, K., Tamiya, E., and Karube, I. (1986). *Anal. Chim. Acta* **188**, 257
- Muramatsu, H., Dicks, J.M., and Karube, I. (1987a). *Anal. Chim. Acta* **197**, 347
- Muramatsu, H., Dicks, J.M., Tamiya, E., and Karube, I. (1987b). *Anal. Chem.* **59**, 2760
- Murray, R.W. (1984). *J. Electroanal. Chem.* **13**, 191
- Nagy, G., von Storp, L.H., and Guilbault, G.G. (1973). *Anal. Chim. Acta* **66**, 443
- Nagy, G., Rice, M.E., and Adams, R.N. (1982). *Life Sci.* **31**, 2611
- Nakako, M., Hanazato, Y., Maeda, M., and Shiono, S. (1986). *Anal. Chim. Acta* **185**, 179
- Nakamoto, S., Kimura, J., and Kuriyama, T. (1987). *GBF Monographs* **10**, 289
- Nakamura, K., Nankai, S., and Iijima, T. (1980). *Natl. Tech. Rep.* **26**, 497
- Nanjo, M., and Guilbault, G.G. (1974a). *Anal. Chem.* **46**, 1769
- Nanjo, M., and Guilbault, G.G. (1974b). *Anal. Chim. Acta* **73**, 367
- Nentwig, J., Scheller, F., Weise, H., and Pfeiffer, D. (1986). *GDR Pat. G 01 N 2778 884*
- Neujahr, H.Y. (1980). *Biotechnol. Bioeng.* **22**, 913
- Neujahr, H.Y. (1982). *Appl. Biochem. Biotechnol.* **7**, 107
- Neujahr, H.Y., and Kjellén, K.G. (1979). *Biotechnol. Bioeng.* **21**, 671
- Newman, A.L., Hunter, K.W., and Stanbro, W.D. (1986). *Proc. 2nd Int. Meeting Chemical Sensors, Bordeaux*, p. 596
- Newman, D.P. (1976). *US Pat.* 3979 274
- Ngeh-Ngwainbi, J., Foley, P.H., Kuan, S.S., and Guilbault, G.G. (1986a). *J. Am. Chem. Soc.* **108**, 5444
- Ngeh-Ngwainbi, J., Foley, P.H., Jordan, J.M., Guilbault, G.G., and Palleschi, G. (1986b). *Proc. 2nd Int. Meeting Chemical Sensors, Bordeaux*, p. 515
- Ngo, T.T. (1987). *Electrochemical Sensors in Immunological Analysis*, Plenum Press, New York
- Ngo, T.T., and Lenhoff, H.M. (1980). *Anal. Lett.* **13**, 1157
- Ngo, T.T., Bovaird, J., and Lenhoff, H.M. (1985). *Appl. Biochem. Biotechnol.* **11**, 63
- Nilsson, H., Akerlund, A.-C., and Mosbach, K. (1973). *Biochim. Biophys. Acta* **320**, 529
- Nilsson, N.J., Mosbach, K., Enfors, S.-O., and Molin, N. (1978). *Biotechnol. Bioeng.* **20**, 527
- Nishikawa, S., Sakai, S., Karube, I., Matsunaga, T., and Suzuki, S. (1982). *Appl. Environ. Microbiol.* **43**, 814
- Niwa, M., Itoh, K., Nagata, A., and Osawa, H. (1981). *Tokai J. Exp. Clin. Med.* **6**, 403
- Noma, A., and Nakayama, K. (1976). *Clin. Chim. Acta* **73**, 487
- Nylander, C., Liedberg, C., and Lund, T. (1982). *Sensors Actuat.* **3**, 79
- Oellerich, M. (1980). *J. Clin. Chem. Clin. Biochem.* **18**, 197
- Ögren, L., and Johansson, G. (1978). *Anal. Chim. Acta* **96**, 1
- Ögren, L., Csiky, L., Risinger, L., Nilsson, L.G., and Johansson, G. (1980). *Anal. Chim. Acta* **117**, 71
- Okada, T., Karube, I., and Suzuki, S. (1982). *Eur. J. Appl. Microbiol. Biotechnol.* **14**, 149
- Okada, T., Karube, I., and Suzuki, S. (1983). *Biotechnol. Bioeng.* **25**, 1641

- Okamoto, M., Sakai, T., and Hayashi, K. (1987). *Biosystems* **21**, 1
- Olsson, B. (1987). Personal communication
- Olsson, B. (1988). *Anal. Chim. Acta* **209**, 123
- Olsson, B., Stalbm, B., and Johansson, G. (1986a). *Anal. Chim. Acta* **179**, 203
- Olsson, B., Lundbäck, H., Johansson, G., Scheller, F., and Nentwig, J. (1986b). *Anal. Chem.* **58**, 1046
- Opitz, N., and Lübbers, D.W. (1987). *GBF Monographs* **10**, 207
- Osakai, T., Katukani, T., and Senda, M. (1988). *Anal. Sci.* **4**, 529
- Osawa, H., Akiyama, S., and Hamada, T. (1981). *Proc. 1st Sensor Symp., Fukuoka*, p. 163
- Özisik, M.N. (1980). *Heat Conduction*, Wiley, New York, chapter 6
- Pacáková, V., Stulik, K., Brabcova, D., and Barthova, J. (1984). *Anal. Chim. Acta* **159**, 71
- Palleschi, G., Rahni, M.A.N., Lubrano, G.J., Ngeh-Ngwainbi, J., and Guilbault, G.G. (1986). *Anal. Biochem.* **159**, 114
- Palleschi, G., Rathore, H., and Mascini, M. (1988). *Anal. Chim. Acta* **209**, 223
- Papariello, G.J., Mukherji, A.K., and Shearer, A.K. (1973). *Anal. Chem.* **45**, 790
- Pascual, C., Pascual, R., and Kotyk, A. (1982). *Anal. Biochem.* **123**, 205
- Pedersen, H., and Horvath, C. (1981). *Appl. Biochem. Bioeng.* **3**, 1
- Petersson, B.A. (1988a). *Anal. Chim. Acta* **209**, 231
- Petersson, B.A. (1988b). *Anal. Chim. Acta* **209**, 239
- Pfeiffer, D., Scheller, F., Jänchen, M., Bertermann, K., and Weise, H. (1980). *Anal. Lett.* **13**, 1179
- Pfeiffer, D., Scheller, F., Schubert, F., and Weise, H. (1987). *Stud. Biophys.* **119**, 183
- Pfeiffer, D., Ralis, E.V., Makower, A., Meiske, C., and Scheller, F. (1990). *J. Chem. Technol. Biotechnol.*, **49**, 255
- Pickup, J.C. (1987). *Proc. 2nd Int. Conf. Bio 87 Sensing and Control*, London, p. 23
- Pilloton, R., Mascini, M., Casella, I., Festo, M., and Bottari, E. (1987). *Anal. Lett.* **20**, 1803
- Pinkerton, T., and Lawson, B. (1982). *Clin. Chem.* **28**, 1946
- Place, J.F., Sutherland, R.M., and Dähne, C. (1985). *Biosensors* **1**, 321
- Planchard, A., Mignot, L., and Junter, G. (1988). *Sensors Actuat.* **14**, 9
- Posadaka, P., and Macholán, L. (1979). *Coll. Czech. Chem. Commun.* **44**, 3395
- Racek, J. (1987). *Anal. Chim. Acta* **197**, 187
- Racek, J., and Musil, J. (1987). *Clin. Chim. Acta* **162**, 129
- Racine, P., Klenk, H.-O., and Kochsiek, K. (1975). *Z. Klin. Chem. Klin. Biochem.* **13**, 533
- Raghavan, K.G., and Ramakrishnan, V. (1986). *Biotechnol. Bioeng.* **28**, 1611
- Raghavan, K.G., Devasagayam, T.P.A., and Ramakrishnan, V. (1986). *Anal. Lett.* **19**, 163
- Rahni, M.A.N., Guilbault, G.G., and de Oliveira, N.G. (1986a). *Anal. Chem.* **58**, 523
- Rahni, M.A.N., Guilbault, G.G., and de Oliveira, N.G. (1986b). *Anal. Chim. Acta* **181**, 219
- Rapoport, S.M. (1977). *Medizinische Biochemie*, 7th Ed., Verlag Volk und Gesundheit, Berlin, p. 127
- Rawson, D.M., Willmer, A.J., and Cardosi, M.F. (1987). *Toxicity Assess.* **2**, 325
- Ray, J., Shinich, T., and Lerner, R. (1979). *Nature* **279**, 215

- Razumas, V.J., Kulys, J.J., and Malinauskas, A.A. (1981). *Environ. Sci. Technol.* **15**, 360
- Razumas, V.J., Jasaitis, J.J., and Kulys, J.J. (1984). *Bioelectrochem. Bioenerg.* **12**, 297
- Rea, P.A., Rolfe, P., and Goddard, P.J. (1985). *Med. Biol. Eng. Comput.* **23**, 108
- Rechnitz, G.A. (1981). *Science* **214**, 287
- Rechnitz, G.A. (1987). *GBF Monographs* **10**, 3
- Rechnitz, G.A., Kobos, R.K., Riechel, T.L., and Gebauer, G.R. (1977). *Anal. Chim. Acta* **94**, 357
- Rechnitz, G.A., Riechel, T.L., Kobos, R.K., and Meyerhoff, M.E. (1978). *Science* **199**, 440
- Rechnitz, G.A., Arnold, M.A., and Meyerhoff, M.E. (1979). *Nature* **278**, 466
- Reitnauer, P.G. (1972). *GDR Pat.* 101 229
- Reitnauer, P.G. (1977). *Z. Med. Lab.-Diagn.* **18**, 60
- Renneberg, R. (1988). Dissertation B, Academy of Sciences of the GDR, Berlin
- Renneberg, R., Pfeiffer, D., Scheller, F., and Jänchen, M. (1982). *Anal. Chim. Acta* **134**, 359
- Renneberg, R., Schössler, W., and Scheller, F. (1983a). *Anal. Lett.* **16**, 1279
- Renneberg, R., Scheller, F., Riedel, K., Litschko, E., and Richter, M. (1983b). *Anal. Lett.* **16**, 877
- Renneberg, R., Riedel, K., Liebs, P., and Scheller, F. (1984). *Anal. Lett.* **17**, 349
- Renneberg, R., Riedel, K., and Scheller, F. (1985). *Appl. Microbiol. Biotechnol.* **21**, 180
- Renneberg, R., Schubert, F., and Scheller, F. (1986). *Trends Biochem. Sci.* **11**, 216
- Richmond, W. (1973). *Clin. Chem.* **19**, 1350
- Riechel, T. L., and Rechnitz, G.A. (1978). *J. Membrane Sci.* **4**, 243
- Riedel, K., and Scheller, F. (1987). *Analyst* **112**, 341
- Riedel, K., Weise, H., Hundertmark, J., and Quade, A. (1984). *Proc. 2nd Heiligenstädter Kolloquium: Wissenschaftliche Geräte für die Biotechnologie* (Lauckner, G., and Beckmann, D., Eds.), Heiligenstadt, p. 333
- Riedel, K., Kühn, M., and Scheller, F. (1985a). *Stud. Biophys.* **107**, 189
- Riedel, K., Liebs, P., and Renneberg, R. (1985b). *J. Basic Microbiol.* **1**, 51
- Riedel, K., Renneberg, R., Liebs, P., and Kaiser, G. (1987). *Stud. Biophys.* **119**, 163
- Riedel, K., Renneberg, R., Kleine, R., Krüger, M., and Scheller, F. (1988). *Appl. Microbiol. Biotechnol.* **38**, 272
- Rigin, V.J. (1978). *Zh. Anal. Khim.* **33**, 1623
- Roberts, G.G. (1983). *Sensors Actuat.* **4**, 131
- Robinson, G.A., Hill, H.A.O., Philo, R.D., Gear, J.M., Rattle, S.J., and Forrest, G.C. (1985). *Clin. Chem.* **31**, 1449
- Robinson, G.A., Martinazzo, G., and Forrest, G.C. (1986a). *J. Immunoassay* **7**, 1
- Robinson, G.A., Cole, V.M., Rattle, S.J., and Forrest, G.C. (1986b). *Biosensors* **2**, 45
- Roederer, J.E., and Bastiaans, G.J. (1983). *Anal. Chem.* **55**, 2333
- Romette, J.L. (1987). *GBF Monographs* **10**, 81
- Romette, J.L., Froment, B., and Thomas, D. (1979). *Clin. Chim. Acta* **95**, 249
- Romette, J.L., Yang, S., Kusakabe, H., and Thomas, D. (1983). *Biotechnol. Bioeng.* **25**, 2557
- Rothen, A. (1947). *J. Biol. Chem.* **186**, 75
- Rothen, A., and Mathot, C. (1971). *Helv. Chim. Acta* **54**, 1208

- Rothen, A., Mathot, C., and Thiele, E.H. (1969). *Experientia* **25**, 420
- Ruslings, J.P., Luttrell, G.H., Cullon, L.R., and Papariello, G.J. (1976). *Anal. Chem.* **48**, 1211
- Ruzicka, J., and Hansen, E. (1974). *Anal. Chim. Acta* **69**, 129
- Santhanam, K., Jespersens, N., and Bard, A.J. (1977). *J. Am. Chem. Soc.* **99**, 274
- Satoh, I., Karube, I., and Suzuki, S. (1976). *Biotechnol. Bioeng.* **18**, 269
- Satoh, I., Karube, I., and Suzuki, S. (1977). *Biotechnol. Bioeng.* **19**, 1095
- Satoh, I., Karube, I., Suzuki, S., and Aikawa, K. (1979). *Anal. Chim. Acta* **106**, 369
- Satoh, I., Danielsson, D., and Mosbach, K. (1981). *Anal. Chim. Acta* **131**, 255
- Schär, H.-P., and Ghisalba, O. (1985). *Biotechnol. Bioeng.* **27**, 897
- Scheler, W. (1985). *Allgemeine und spezielle Pharmakologie* (Markwardt, F., Ed.), 5th Ed. Verlag Volk und Gesundheit, Berlin
- Scheller, F., and Karsten, C. (1983). *Anal. Chim. Acta* **155**, 29
- Scheller, F., and Pfeiffer, D. (1978). *Z. Cem.* **18**, 50
- Scheller, F., and Strnad, G. (1982). *Adv. Chem. Ser.* **201**, 219
- Scheller, F., Pfeiffer, D., Seyer, I., Kirstein, D., Schulmeister, Th., and Nentwig, J. (1983a). *Bioelectrochem. Bioenerg.* **11**, 155
- Scheller, F., Wollenberger, U., Schubert, F., Pfeiffer, D., Renneberg, R., Jänchen, M., Walzel, G., Weise, H., and Bertermann, K. (1983b). *Priklad. Biokhim. Mikrobiol.* **18**, 454
- Scheller, F., Siegbahn, N., Danielsson, B., and Mosbach, K. (1985a). *Anal. Chem.* **57**, 1740
- Scheller, F., Schubert, F., Renneberg, R., Müller, H.-G., Jänchen, M., and Weise, H. (1985b). *Biosensors* **1**, 135
- Scheller, F., Schubert, F., Olsson, B., Gorton, L., and Johansson, G. (1986a). *Anal. Lett.* **19**, 1691
- Scheller, F., Pfeiffer, D., Kühn, M., Hamann, H., Fahrenbruch, B., Klimes, N., Nentwig, J., Möricke, R., Kiesewetter, M., Jänchen, M., Scholz, P., Müller, E., Uffrecht, E., Brunner, J., and Hanke, G. (1986b). *Z. Klin. Med.* **41**, 565
- Scheller, F., Schubert, F., Pfeiffer, D., and Renneberg, R. (1987a). *Enzyme Eng.* **8**, 240
- Scheller, F., Wollenberger, U., Schubert, F., Pfeiffer, D., and Bogdanovskaya, V.A. (1987b). *GBF Monographs* **10**, 39
- Scheller, F., Kirstein, D., Kirstein, L., Schubert, F., Wollenberger, U., Olsson, B., Gorton, L., and Johansson, G. (1987c). *Phil. Trans. R. Soc. London* **316B**, 85
- Scheller, F., Renneberg, R., and Schubert, F. (1988). *Methods Enzymol.* **137 D**, 29
- Scheller, F., Pfeiffer, D., Hintsche, R., Schubert, F., and Wollenberger, U. (1989). *GBF Monographs*, **13**, 3
- Scheller, F., Hintsche, R., Neumann, B., and Bogdanovskaya, V. (1989b). *Stud. Biophys.* **132**, 93
- Schelter-Graf, A., Schmidt, H.-L., and Huck, H. (1984). *Anal. Chim. Acta* **163**, 299
- Schindler, J.G., and Schindler, M.M. (1983). *Bioelektrochemische Membranelektroden*, Walter de Gruyter, Berlin, New York
- Schindler, J.G., and von Gülich, M. (1981). *Fres. Z. Anal. Chem.* **308**, 434
- Schläpfer, P., Mindt, W., and Racine, P. (1974). *Clin. Chim. Acta* **57**, 283
- Schmid, R.D. (1985). *Appl. Microbiol. Biotechnol.* **22**, 157
- Schmidt, H., Kirsam, G., and Grenner, G. (1976). *Biochim. Biophys. Acta* **429**, 283
- Schubert, F. (1983). *Dissertation*, Academy of Sciences of the GDR, Berlin

- Schubert, F., and Scheller, F. (1983). GDR Pat. 228 825
- Schubert, F., and Scheller, F. (1988a). *Methods Enzymol.* **137 D**, 152
- Schubert, F., and Scheller, F. (1988b). GDR Pat. C 12 Q/315 7391
- Schubert, F., and Weigelt, D. (1986). Unpublished results
- Schubert, F., Kirstein, D., Scheller, F., and Mohr, P. (1980). *Anal. Lett.* **13**, 1167
- Schubert, F., Scheller, F., and Kirstein, D. (1982a). *Anal. Chim. Acta* **141**, 15
- Schubert, F., Scheller, F., Mohr, P., and Scheler, W. (1982b). *Anal. Lett.* **15**, 681
- Schubert, F., Wollenberger, U., and Scheller, F. (1983). *Biotechnol. Lett.* **5**, 239
- Schubert, F., Renneberg, R., Scheller, F., and Kirstein, L. (1984). *Anal. Chem.* **56**, 1677
- Schubert, F., Kistein, D., Schröder, K.-L., and Scheller, F. (1985a). *Anal. Chim. Acta* **169**, 391
- Schubert, F., Kistein, D., Scheller, F., Abraham, M., and Boross, L. (1985b). *Acta Biotechnol.* **5**, 375
- Schubert, F., Kistein, D., Scheller, F., Abraham, M., and Boross, L. (1986a). *Anal. Lett.* **19**, 2155
- Schubert, F., Kirstein, D., Scheller, F., Appelqvist, R., Gorton, L., and Johansson, G. (1986b). *Anal. Lett.* **19**, 1273
- Schubert, F., Scheller, F., and Krasteva, N.G. (1990). *Electroanalysis*, **2**, 347
- Schubert, F., Wollenberger, U., Scheller, F., and Müller, H.-G. (1991). In: *Biosensors and Bioinstrumentation* (Wise, D.L., Ed.), Marcel Dekker, New York, p. 19
- Schulmeister, Th. (1987a). *Anal. Chim. Acta* **198**, 223
- Schulmeister, Th. (1987b). *Anal. Chim. Acta* **201**, 305
- Schulmeister, Th., and Scheller, F. (1985a). *Anal. Chim. Acta* **171**, 111
- Schulmeister, Th., and Scheller, F. (1985b). *Anal. Chim. Acta* **170**, 279
- Schultz, J., and Sims, G. (1979). *Biotechnol. Bioeng. Symp.* **9**, 65
- Seegopaul, P., and Rechnitz, G.A. (1983). *Anal. Chem.* **55**, 1929
- Seifert, M., Tiefenthaler, K., Heuberger, K., Lukosz, W., and Mosbach, K. (1986). *Anal. Lett.* **19**, 205
- Seitz, R. (1984). *Anal. Chem.* **56**, 16A
- Seitz, R., Cole, T., and Mullin, J. (1982). 11th Int. Congr. Clinical Chemistry, Walter de Gruyter, Berlin, New York, p. 1083
- Senda, M. (1988). *Abstr. Int. BioSymposium, Nagoya*, p. 65
- Shaojun, D., Baifeng, L., and Jun, B. (1985). *Sci. Sinica Ser. B* **28**, 13
- Shichiri, M. (1987). *GBF Monographs* **10**, 95
- Shichiri, M., Kawamori, R., Hakui, N., Asakawa, N., Yamasaki, Y., and Abe, H. (1984). *Biomed. Biochim. Acta* **43**, 561
- Shimura, T., Nakamura, T., Kawakami, A., Haga, M., and Kato, Y. (1986). *Chem. Pharm. Bull.* **34**, 5020
- Shinbo, T., Sugiura, M., and Kamo, N. (1979). *Anal. Chem.* **51**, 100
- Shiono, S., Hanazato, Y., and Nakako, M. (1986). *Anal. Sci.* **2**, 517
- Shiono, S., Hanazato, Y., Nakako, M., and Maeda, M. (1987). *GBF Monographs* **10**, 291
- Shons, A., Dorman, F., and Najarian, J. (1972). *J. Biomed. Mater. Res.* **6**, 565
- Shu, R., and Wilson, G.S. (1976). *Anal. Chem.* **48**, 1679
- Sidwell, J.S., and Rechnitz, G.A. (1985). *Biotechnol. Lett.* **7**, 419
- Sidwell, J.S., and Rechnitz, G.A. (1986). *Biosensors* **2**, 221

- Silver, I.A. (1976). In: *Ion and Enzyme Electrodes in Biology and Medicine* (Kessler, M., Clark, L.C., Lübberts, D., Silver, I.A., and Simon, W., Eds.), Urban und Schwarzenberg, München, p. 189
- Simon, W. (1987). *GBF Monographs* **10**, 13
- Smit, N., and Rechnitz, G.A. (1984). *Biotechnol. Lett.* **6**, 209
- Smith, A.G., and Brooks, C.J.W. (1976). *J. Steroid Biochem.* **7**, 705
- Smith, G.D. (1965). *Numerical Solution of Partial Differential Equations*, Oxford University Press, London
- Smith, V.J. (1987). *Anal. Chem.* **59**, 2259
- Solsky, R.L., and Rechnitz, G.A. (1981). *Anal. Chim. Acta* **123**, 135
- Sonawat, H.M., Phadke, R.S., and Govil, G. (1984). *Biotechnol. Bioeng.* **26**, 1066
- Soutter, W.P., Sharp, F., and Clark, D.M. (1978). *Brit. J. Anaesth.* **40**, 445
- Srinivasan, K.R., Mansouri, S., and Schulz, J.S. (1986). *Biotechnol. Bioeng.* **28**, 233
- Stanley, C.J., Paris, F., Plumb, A., Webb, A., and Johansson, A. (1985). *Int. Biotechnol. Lab.* **3**, 46
- Stenberg, M., and Nygren, H.N. (1982). *Anal. Biochem.* **127**, 183
- Stenberg, R., Bindner, D., Welsin, G., and Thevenot, D. (1988). *Anal. Chem.* **60**, 2781
- Stefanac, Z. and Simon, W. (1966). *Chimia* **20**, 436
- Sternson, L.A. (1974). *Anal. Chem.* **46**, 2228
- Stow, R., and Randall, B. (1973). *Am. J. Physiol.* **33**, 97
- Strand, S.E., and Carlson, D.A. (1984). *J. Water Pollut. Contr. Fed.* **56**, 464
- Strassner, W. (1980). *Laborwerte und ihre klinische Bedeutung*, 4th Ed., Verlag Volk und Gesundheit, Berlin
- Strnad, G. (1989). *Dissertation*, Humboldt University, Berlin
- Suaud-Chagny, M., and Goup, F. (1986). *Anal. Chem.* **58**, 412
- Suaud-Chagny, M., and Pujol, J. (1985). *Analisis* **13**, 25
- Sugawara, M., Kojima, K., Sazawa, H., and Umezawa, Y. (1987). *Anal. Chem.* **59**, 2842
- Sundaram, P., and Jayonne, B. (1979). *Clin. Chim. Acta* **94**, 309
- Sutherland, R.M., Dähne, C., and Place, J.F. (1984). *Anal. Lett.* **17**, 43
- Sutherland, R.M., Frevert, J., Place, J.F., Kuoeil, H., Begnard, A., Dähne, C., Revillet, G., and Hybl, E. (1987). *GBF Monographs* **10**, 305
- Suva, R., Rimer, V., Brandt, S., Madou, M., and Ross, R. (1986). *Proc. 2nd Int. Meeting Chemical Sensors*, Bordeaux, p. 542
- Suzuki, H., Tamiya, E., and Karube, I. (1988a). *Anal. Chem.* **60**, 1078
- Suzuki, H., Tamiya, E., Karube, I., and Oshima, T. (1988b). *Anal. Lett.* **21**, 1323
- Suzuki, S., and Karube, I. (1979). *Ann. N. Y. Acad. Sci.* **326**, 255
- Suzuki, S., and Karube, I. (1980). *Proc. 6th Fermentation Symp., London (Canada)*, vol. 3, p. 355
- Szuminski, M., Chen, A., and Liu, C.C. (1984). *Biotechnol. Bioeng.* **26**, 642
- Tabata, M., and Murachi, T. (1983). *Biotechnol. Bioeng.* **25**, 3013
- Tabata, M., Endo, J., and Murachi, T. (1981). *J. Appl. Biochem.* **3**, 84
- Takatsu, I., and Morizuma, T. (1987). *Sensors Actuat.* **11**, 309
- Tamiya, E., Karube, I., Kitagawa, Y., Ameyama, N., and Nakashima, K. (1988). *Anal. Chim. Acta* **207**, 77
- Taniguchi, J., and Hawkridge, F. (1988). *J. Electroanal. Chem.* **240**, 333

- Tarasevich, M.R. (1985). In: *Comprehensive Treatise of Electrochemistry* (Srinivasan, S., Chizmadzhev, Yu., Bockris, J.O.M., Conway, B.E., and Yeager, E., Eds.), Plenum Press, New York, London, p. 231
- Thevenot, D.R. (1982). *Diabetes Care* **5**, 184
- Thevenot, D.R., Sternberg, R., Coulet, P.R., Laurent, J., and Gautheron, D.C. (1979). *Anal. Chem.* **51**, 96
- Thevenot, D.R., Sternberg, R., and Coulet, P.R. (1982). *Diabetes Care* **5**, 203
- Thompson, M. (1987). *GBF Monographs* **10**, 145
- Thompson, M., Krull, U.J., and Bendell-Young, L.J. (1983). *Talanta* **30**, 919
- Thompson, M., Dorn, W.H., Krull, U.J., Tauskela, J.S., Vanderberg, E.T., and Wong, H. (1986). *Anal. Chim. Acta* **180**, 251
- Tijssen, P. (1985). *Practice and Theory of Enzyme Immuno assay*, Elsevier, Amsterdam
- Tokinaga, D., Kobayashi, T., Katori, A., Karasawa, Y., and Yasuda, K. (1984). *Proc. Int. Meeting Chemical Sensors*, Fukuoka, Elsevier, Amsterdam, p. 626
- Torstensson, A., Johansson, G., Mansson, M.-O., Larsson, P., and Mosbach, K. (1980). *Anal. Lett.* **13**, 837
- Toul, Z., and Macholán, L. (1975). *Coll. Czech. Chem. Commun.* **40**, 2208
- Toyota, T., Kuan, S., and Guilbault, G.G. (1985). *Anal. Chem.* **57**, 1925
- Tran-Minh, C., and Beaux, J. (1979). *Anal. Chem.* **51**, 92
- Tran-Minh, C., and Broun, G. (1975). *Anal. Chem.* **47**, 1359
- Tran-Minh, C., and Vallin, D. (1978). *Anal. Chem.* **50**, 1874
- Tran-Minh, C., Yamani, H., and Abdul, M. (1986). *Proc. 2nd Int. Meeting Chemical Sensors*, Bordeaux, p. 615
- Traylor, P., Kmetec, E., and Johnson, J. (1977). *Anal. Chem.* **49**, 789
- Trettnak, W., Leiner, M.J.P., and Wolfbeis, O. (1989). *Biosensors* **4**, 15
- Tschannen, R. (1988). *Alimenta* **6**, 152
- Tschannen, R., Hoeren, R., and Schröder, N. (1987). *Biotechnologie* **2**, 18
- Tsuchida, T., and Yoda, K. (1981). *Enzyme Microb. Technol.* **3**, 326
- Tsuchida, T., and Yoda, K. (1982). *J. Chem. Soc. Japan*, 1361
- Tsuchida, T., and Yoda, K. (1983). *Clin. Chem.* **29**, 51
- Tsuchida, T., Takasugi, H., Yoda, K., Takizawa, K., and Kobayashi, S. (1985). *Biotechnol. Bioeng.* **27**, 837
- Turner, A.P.F. (1985). *Proc. Biotech Europe*, Online Publications, Pinner, p. 181
- Turner, A.P.F., Aston, W.J., Higgins, I.J., Bell, J., Colby, J., Davis, G., and Hill, H.A.O. (1984). *Anal. Chim. Acta* **163**, 161
- Turner, A.P.F., D'Costa, E.J., and Higgins, I.J. (1987a). *Enzyme Eng.* **8**, 281
- Turner, A.P.F., Hendry, S.P., and Cardosi, M.F. (1987b). *Biotech World Rep.* **1**, 125
- Uchiyama, S., and Rechnitz, G.A. (1987). *Anal. Lett.* **20**, 451
- Uchiyama, S., Sato, Y., Tofoku, Y., and Suzuki, S. (1987). *J. Electrochem. Soc.* **134**, 501C
- Uchiyama, S., Sato, Y., Tofoku, Y., and Suzuki, S. (1988a). *Anal. Chim. Acta* **209**, 351
- Uchiyama, S., Tamata, M., Tofoku, Y., and Suzuki, S. (1988b). *Anal. Chim. Acta* **208**, 287
- Umana, M., and Waller, J. (1983). *Proc. Int. Meeting Chemical Sensors*, Fukuoka, Elsevier, Amsterdam, p. 705
- Umezawa, Y., Sofue, S., and Takamoto, Y. (1982). *Anal. Lett.* **15**, 135

- Umezawa, Y., Kataoka, M., Sugawara, M., Abe, H., Kojima, K., Tokinami, M., Sazawe, H., and Yasuda, Y. (1987). *GBF Monographs* **10**, 139
- Updike, S.J., and Hicks, G.P. (1967). *Nature* **214**, 986
- Updike, S.J., and Treichel J. (1979). *Anal. Chem.* **51**, 1643
- Uwajima, T., Shimizu, Y., Terada, O. (1984). *J. Biol. Chem.* **259**, 2748
- Vadgama, P.M., Alberti, K.G.M.M., and Covington, A.K. (1982). *Anal. Chim. Acta* **136**, 403
- Van der Schoot, B.H., and Bergveld, P. (1987). *Anal. Chim. Acta* **199**, 157
- Van der Schoot, B.H., and Bergveld, P. (1987/88). *Biosensors* **3**, 161
- Varfolomeev, S.D., and Bachurin, S.O. (1984). *J. Mol. Catal.* **27**, 305
- Varfolomeev, S.D., and Berezin, I.V. (1978). *J. Mol. Catal.* **4**, 387
- Varfolomeev, S.D., Bachurin, S.O., and Nagui, A. (1980). *J. Mol. Catal.* **9**, 223
- Verduyn, C., van Dijken, J., and Scheffers, W. (1983). *Biotechnol. Bioeng.* **25**, 1049
- Verduyn, C., Zomerdijk, T., van Dijken, J., and Scheffers, W. (1984). *Appl. Microbiol. Biotechnol.* **19**, 181
- Vidziunaite, R., and Kulys, J.J. (1985). *Trud. Akad. Nauk Lit. SSR Ser. C* **2(90)**, 84
- Vincké, B.J., Devleeschouwer, M.J., and Patriarche, G.J. (1983a). *J. Pharm. Belg.* **38**, 225
- Vincké, B.J., Devleeschouwer, M.J., and Patriarche, G.J. (1983b). *Anal. Lett.* **16**, 673
- Vincké, B.J., Devleeschouwer, M.J., Dony, J., and Patriarche, G.J. (1984). *Int. J. Pharm.* **21**, 265
- Vincké, B.J., Devleeschouwer, M.J., and Patriarche, G.J. (1985a). *Anal. Lett.* **18**, 593
- Vincké, B.J., Devleeschouwer, M.J., and Patriarche, G.J. (1985b). *J. Pharm. Belg.* **40**, 357
- Vincké, B.J., Devleeschouwer, M.J., and Patriarche, G.J. (1985c). *Anal. Lett.* **18**, 1593
- Vorberg, S., and Schöpp, W. (1985). *Fres. Z. Anal. Chem.* **320**, 48
- Walters, R.R., Moriarty, B.E., and Buck, R.P. (1980). *Anal. Chem.* **52**, 1680
- Wang, J., and Lin, M.S. (1988). *Anal. Chem.* **60**, 1545
- Wang, J., and Lin, M.S. (1989). *Electroanalysis* **1**, 43
- Wangsa, J., and Arnold, M. (1988). *Anal. Chem.* **60**, 1050
- Wasa, T., Akimoto, K., Ueda, K., and Yao, T. (1984a). *Bunseki Kagaku* **33**, 471
- Wasa, T., Akimoto, K., Yao, T., and Murao, S. (1984b). *Nippon Kagaku Kaishi* **9**, 1397
- Watanabe, E., Ogura, T., Toyama, K., Karube, I., Matsuoka, H., and Suzuki, S. (1984). *Enzyme Microb. Technol.* **6**, 207
- Watanabe, E., Endo, H., Hayashi, T., and Toyama, K. (1986). *Biosensors* **2**, 235
- Watanabe, E., Endo, H., and Toyama, K. (1987/88). *Biosensors* **3**, 297
- Watson, B., and Keyes, M. (1976). *Anal. Lett.* **9**, 713
- Watson, L.D., Maynard, P., Cullen, D.C., Sethi, R.S., Brettle, J., and Lowe, C.R. (1987/88). *Biosensors* **3**, 101
- Weaver, J., Cooney, C., Fulton, S., Schüler, S., and Tannenbaum, S. (1976). *Biochim. Biophys. Acta* **452**, 285
- Weaver, M.R., and Vadgama, P.M. (1986). *Clin. Chim. Acta* **155**, 295
- Weber, S.G., and Purdy, W.C. (1979). *Anal. Lett.* **12**, 1
- Weetall, H.H. (1976). *Methods Enzymol.* **44**, 134
- Wehmeyer, K.R., Halsall, H.B., and Heineman, W.R. (1982). *Clin. Chem.* **28**, 1968
- Wehmeyer, K.R., Halsall, H.B., Heineman, W.R., Volle, C.P., and Chen, J.W. (1986). *Anal. Chem.* **58**, 135

- Wehnert, G., Sauerbrei, A., Bayer, T., Scheper, T., and Schügerl, K. (1987). *Anal. Chim. Acta* **200**, 73
- Weigelt, D. (1987). Dissertation, Humboldt-University, Berlin
- Weigelt, D., Schubert, F., and Scheller, F. (1987a). *Analyst* **112**, 1155
- Weigelt, D., Schubert, F., and Scheller, F. (1987b). *Fres. Z. Anal. Chem.* **328**, 259
- Weigelt, D., Schubert, F., and Scheller, F. (1988). *Anal. Lett.* **21**, 225
- Weil, M.H., Leavy, J.A., Rackow, E.C., Halfman, C.J., and Bruno, S.J. (1986). *Clin. Chem.* **32**, 2175
- Weise, H., and Scheller, F. (1979). *Lebensmittelind.* **26**, 206
- Weise, H., and Scheller, F. (1981). *Lebensmittelind.* **28**, 491
- Weise, H., Kreibich, G., and Scheller, F. (1987). *Acta Biotechnol.* **7**, 61
- Welin, S., Elwing, H., Arwin, H., Lundström, I., and Wikström, M. (1984). *Anal. Chim. Acta* **163**, 263
- Wieck, H., Heider, G.H., and Yacynych, A.M. (1984). *Anal. Chim. Acta* **158**, 137
- Williams, D.L., Doig, A.R., and Korosi, A. (1970). *Anal. Chem.* **42**, 118
- Wingard, L.B. (1983). *Fed. Proc.* **42**, 288
- Wingard, L.B. (1984). *Trends Anal. Chem.* **3**, 235
- Wingard, L.B. (1987). *GBF Monographs* **10**, 133
- Wingard, L.B., Liu, C.C., Wolfson, S.K., Yao, S.J., and Drash, A.L. (1982). *Diabetes Care* **5**, 199
- Winquist, F., Spetz, A., Lundström, I., and Danielsson, B. (1984). *Anal. Chim. Acta* **163**, 143
- Winquist, F., Spetz, A., Armgarth, M., Lundström, I., and Danielsson, B. (1985). *Sensors Actuat.* **8**, 91
- Winquist, F., Lundström, I., and Danielsson, B. (1986). *Anal. Chem.* **58**, 145
- Wolf, E., and Zschiesche, A. (1986). *Z. Med. Lab.-Diagn.* **27**, 130
- Wolfbeis, O.S. (1986). *Fres. Z. Anal. Chem.* **325**, 387
- Wolfbeis, O.S. (1987). *GBF Monographs* **10**, 197
- Wolfbeis, O.S. (1989). *Appl. Fluorescence Technol.*, **1**, 1
- Wollenberger, U. (1981). Unpublished results
- Wollenberger, U. (1984). Dissertation, Academy of Sciences of the GDR, Berlin
- Wollenberger, U., Scheller, F., and Atrat, P. (1980a). *Anal. Lett.* **13**, 825
- Wollenberger, U., Scheller, F., and Atrat, P. (1980b). *Anal. Lett.* **13**, 1201
- Wollenberger, U., Kühn, M., Scheller, F., Deppmeyer, V., and Jänchen, M. (1983). *Bioelectrochem. Bioenerg.* **11**, 307
- Wollenberger, U., Scheller, F., Pfeiffer, D., Bogdanovskaya, V., Tarasevich, M.R., and Hanke, G. (1986). *Anal. Chim. Acta* **187**, 39
- Wollenberger, U., Schubert, F., Scheller, F., Danielsson, B., and Mosbach, K. (1987a). *Stud. Biophys.* **119**, 167
- Wollenberger, U., Schubert, F., Scheller, F., Danielsson, B., and Mosbach, K. (1987b). *Anal. Lett.* **20**, 657
- Wollenberger, U., Scheller, F., Böhmer, A., Passarge, M., and Müller, H.-G. (1989). *Biosensors* **4**, 381
- Wortberg, B. (1975). *Z. Lebensm. Unters. Forsch.* **157**, 333
- Wrighton, M. (1986). *Science* **231**, 32
- Wrighton, M., Thackeray, J., Natan, M., Smith, D., Lane, G., and Belanger, D. (1987). *Phil. Trans. R. Soc. London* **316B**, 3

- Yacynych, A.M., Sasso, S.V., Reynolds, E.R., and Geise, R.J. (1987). GBF Monographs **10**, 69
- Yalow, R.S., and Berson, S.A. (1959). *Nature* **184**, 1648
- Yamamoto, N., Nagasawa, Y., Sawai, M., Sudo, T., and Tsubomura, H. (1978). *J. Immunol. Methods* **22**, 309
- Yamamoto, Y., Nagaoka, S., Tamaka, T., Shiro, T., Honma, K., and Tsubomura, H. (1983). *Proc. Int. Meeting Chemical Sensors, Fukuoka, Elsevier, Amsterdam*, p. 699
- Yamauchi, H., Kusakabe, H., Midorikawa, Y., Fujishima, T., and Kuninaka, A. (1983). *Proc. Int. Congr. Biotechnology, München*, p. I-705
- Yang, J.S. (1986). *Haniguk Saenghura Hakkoichi* **19**, 13
- Yao, S., Wolfson, S., and Tokarsky, J. (1975). *Bioelectrochem. Bioenerg.* **2**, 348
- Yao, T. (1983). *Anal. Chim. Acta* **153**, 169
- Yao, T., and Musha, S. (1979). *Anal. Chim. Acta* **110**, 203
- Yao, T., and Wasa, T. (1988a). *Anal. Chim. Acta* **209**, 259
- Yao, T., and Wasa, T. (1988b). *Anal. Chim. Acta* **207**, 319
- Yoda, K. (1988). *Methods Enzymol.* **137 D**, 61
- Yoda, K., and Tsuchida, T. (1983). *Proc. Int. Meeting Chemical Sensors, Fukuoka, Elsevier, Amsterdam*, p. 648
- Yoshino, F., and Osawa, H. (1980). *Clin. Chem.* **26**, 1060
- Yuan, C.-L., Kuan, S.S., and Guilbault, G.G. (1981). *Anal. Chim. Acta* **124**, 169

This Page Intentionally Left Blank

Subject Index

- Acetylcholine esterase, 182, 183, 256, 262, 287, 296, 307, 320
- Acid phosphatase, 250, 296
- Activation energy, 37, 64–66
- Activity of immobilized enzymes, 53–56
- Acyl CoA synthetase, 212
- Adenosine monophosphate, 211, 212, 249
- Affinity sensors, 244–281
 - definition, 8, 9
 - overview, 256
- Alanine aminopeptidase, 308
- Alanine aminotransferase, 201–203, 310
- Albumin determination, 16, 253, 266, 267, 275, 276, 283, 284
- Alcohol dehydrogenase, 136–138, 230, 253, 270
- Alcohol determination, 4, 136–138, 317, 319
 - using analyzers, 295, 317
 - using a microbial field effect transistor, 244
 - using substrate recycling, 137, 223
- Alcohol oxidase, 136, 137, 295, 317, 319
 - alcohol dehydrogenase electrode, 137, 223
- Alkaline phosphatase, 15, 38
 - apoenzyme electrode, 260
 - determination of, 142, 230
 - glucose oxidase electrode, 198, 261
 - as marker enzyme, 259, 269, 270
- Aminopyrine, 143
- Amino acids, 157–159, 249, 322
- Amino acid oxidase, 157, 158
 - electrochemical conversion of, 33
- Amperometric electrodes, 24–34
 - detection limit of, 24
 - selectivity of, 24, 27
- Amplification, 78–80, 220–230, 270, 271
 - factor, 80, 222–227
- α -Amylase, 243, 295, 308
- Analyzers using enzyme electrodes, 295–310
- Aniline, 214, 215, 233, 234
- Antibody, 5, 8
- Antibody–antigen complex formation, 48, 49
- Antigen, 5, 8
- Anti-interference layer, 186, 216–220
 - as switch, 325
 - for ascorbate, 219
 - for glucose, 217, 218
 - for lactate, 218
 - for oxygen, 220
- Apoenzyme, 8
 - electrode, 185, 259, 260
- Apyrase, 294
- Arrhenius plot, 66
- Arylsulphatase, 261
- Ascorbate oxidase, 150, 152, 294
 - anti-interference layer, 219
- Ascorbic acid, 150, 152, 249, 294, 322
 - eliminator electrode for, 30
- Aspartame, 242, 243
- Aspartate aminotransferase, 38, 203, 310
- Assimilation test, 245, 248
- ATP determination
 - using competition sensors, 212–214
 - using substrate recycling, 222, 224, 227, 228
 - using an organelle sensor, 233, 236
 - using a thermistor, 294
- Avidin, 256–258
- B-Lymphocytes, 256, 258
- Banana sensor, 249
- Bilayer lipid membrane, 106, 252, 287, 289
- Bile acids, 148
- Bilirubin, 143, 196
- Biochemically modified electrodes, 31

- Biocomputer, 325
- Bioelectronics, 6, 323–325
 - Biogenic amines, 143
 - Biological Oxygen Demand, 245–247, 322
- Bioluminescence, 16
- Biomimetic sensors, 9
- Biosensor, 3–10
 - configuration, 7
 - generations of, 9, 10
 - linear range of, 61–64
 - detection limit of, 61–64
 - market for, 291
- Biotin, 256–258
- Blue crab antennule sensor, 288, 289
- Butyrylcholine esterase, 262, 263

- Calorimetric biosensors, 10–13
- Carbon monoxide determination, 155, 156, 262
- Carbon monoxide oxidoreductase, 155, 156
- Carnitine, 143
- Carnitine dehydrogenase–diaphorase sensor, 143
- Catalase, 11, 294, 320
 - as marker enzyme, 256, 275–277
- Catechol, 142
 - Catechol oxygenase, 140, 142
- Catecholamines, 152, 217
- Cell number, 245, 247, 248
- Cell populations, 245
- Cellulases, 184
- Cellobiose, 320
- Cephalosporin, 320
- Ceruloplasmin, 143
- Chemically modified electrodes
 - preparation of, 30–34
- Chemoreceptor, 2, 3, 7, 8, 10
 - sensor using, 252, 287–289
- Chloroperoxidase, 268
- Cholestenone, 148, 262
- Cholesterol determination, 144–148, 294
 - using enzyme reactors, 204, 205
 - using enzyme sequence electrodes, 205–207
 - using optical sensors, 16
- Cholesterol ester hydrolase, 38, 144, 204–206
- Cholesterol oxidase, 11, 60, 144, 262, 294
 - electrochemical conversion of, 33
- Choline, 207–209
- Choline oxidase, 207, 208, 294
 - acetylcholine esterase electrode, 207
- Cholinesterase, 262, 263, 307
 - inhibitors of, 308
- Chorionic gonadotrophin, 276, 277
- Chymotrypsin, 159
- Coenzymes, 39–41
- Cofactors, 41
 - Competition, 8, 185, 212–214
 - Concanavalin A, 255–257
- Conductometric sensors, 34
- Coupling of enzyme reactions, 5, 8, 184–186, 323–325
- Creatine, 209
- Creatine amidinohydrolase, 60, 209
- Creatinine, 174–176, 209, 210, 216, 294
- Creatinine amidohydrolase, 60, 175, 209
- Creatinine iminohydrolase, 175, 176
- Creatine kinase, 202, 272, 296
- Cu²⁺ determination, 260
- Cyanide, 320
- Cyclic substrate conversion, 78–81, 220–229
- Cytochrome b₂, 127, 129, 130, 304
 - electrochemical conversion of, 33
 - laccase electrode, 223–225
 - lactate dehydrogenase electrode, 222, 227
- Cytochrome c, 33, 130, 155
- Cytochrome oxidase, 155
- Cytochrome P-450
 - electrochemical conversion of, 33
 - immobilization, 57
 - sensors, 232–236

- Dehydrogenase determination, 253
- Diamine oxidase, 143
- Diaphorase, 29, 143, 270, 272
- Digoxin, 268
- Dinitrophenol, 268, 280–282
- Direct electron transfer, 31–34
- Disposable biosensors, 291, 292

- DNA hybridization test, 259
- Double recycling sensor, 229
- ECA 20, 295, 300, 301, 306
- Effectiveness factor, 57
- Electrochemically converted proteins, 33
- Electrodes, 8, 18–34
 - amperometric, 24–34
 - conductometric, 34
 - potentiometric, 18–24
- Elimination of interferences, 214–220, 302
- Eliminator electrode, 152
- Ellipsometry, 17, 285
- Enthalpy of enzyme catalyzed reactions, 11
- Enzyme activity, 45
 - apparent, 56, 60
 - determination of, 307–311
 - recovery of, 56, 57
- Enzyme competition electrode, 212–214, 324
 - modeling of, 75–78
- Enzyme immunoassay, 48, 264–271
- Enzyme immunoelectrode, 275–283
- Enzyme kinetics, 41–45
- Enzyme loading test, 59
- Enzymes, 35–48
 - classification of, 37–39
 - pH dependence of, 46
 - structure of, 35
 - temperature dependence of, 46
- Enzyme sequence electrode, 186–212, 323, 324
 - modeling of, 73–75
- Enzyme stirrer, 90, 138, 161, 204, 205
 - Enzyme thermistor, 10–13, 89, 90, 127, 149, 162, 174, 183, 197, 293, 294, 320
- ESAT 6660, 295, 300, 301, 306
- Estriol, 270
 - Ethanol, 136–138, 317, 319
- Externally buffered enzyme electrode, 97
- FAD determination, 259
- Ferrocene, 30, 88, 111–114, 121, 269, 302, 303, 312
- a-Fetoprotein, 276, 277
- Fiber optic sensor, 13–16, 256
- Field effect transistor, 5, 8, 20–24
- Flower tissue sensors, 249, 251
- Fluoride, 249, 251, 264
- Fluorimetry, 13–16
- Formaldehyde, 256, 259
- Formaldehyde dehydrogenase, 256, 259
- Fructose, 197–199, 213, 214, 238, 244
- Galactose, 125, 244, 295, 320
- Galactose oxidase, 125, 320
- β -Galactosidase, 60, 121, 296, 320
 - glucose oxidase electrode, 192, 317
- Glucoamylase, 38, 296
 - glucose oxidase electrode, 76, 191, 192
- Gluconate, 126, 186
- Gluconate dehydrogenase, 126
- Gluconolactonase, 195
- Glucosamine-6-phosphate, 248–250
- Glucose-converting enzymes, 87, 88
- Glucose dehydrogenase, 88, 90, 93
 - horseradish peroxidase electrode, 222
- Glucose determination, 85–125, 296–303, 311–313, 316–322
 - with affinity sensors, 255, 256
 - with amperometric microelectrodes, 118–121
 - with automatic analyzers, 100–103, 295–303, 316–322
 - in blood, 295–303
 - in cell cultures, 320–322
 - with chemically modified electrodes, 107–117
 - with enzyme electrodes, 56–65, 90–107, 197–199
 - with enzyme reactors, 88–90, 216
 - with an enzyme stirrer, 90
 - with enzyme thermistors, 90, 106, 107
 - in fermentation, 97, 316–320
 - with FIA, 90, 104, 108, 194, 216
 - with field effect transistors, 121–125
 - with the Glukometer, 103, 296–299, 311, 316
 - with implantable sensors, 311–313
 - in meat, 113

- with a microbial field effect transistor, 238
- with microelectrodes, 118–120
- with optical sensors, 15, 106, 107, 256, 257, 292
- using substrate recycling, 222, 227
- with test strips, 3, 292
- in urine, 301, 302
- Glucose isomerase, 38
 - glucose oxidase electrode, 197
- Glucose oxidase, 3, 11, 26, 37, 87
 - apparent K_M value, 60
 - catalase electrode, 197
 - antiinterference layer, 217, 218, 220
 - characteristics of, 87
 - coupling with other enzymes, 184
 - electrochemical conversion, 33
 - electrodes, 90–117
 - characterization of, 56–65
 - modeling of, 70–72
 - pH dependence of, 64
 - gluconolactonase electrode, 195, 196
 - glucose dehydrogenase electrode, 212, 213, 222, 227
 - glucose isomerase electrode, 197
 - hexokinase electrode, 213
 - horseradish peroxidase electrode, 92, 143, 195, 196
 - invertase electrode, 187–190
 - as marker enzyme, 268, 276–278
 - membrane preparation, 92, 95, 99, 100, 103, 112
 - myrosinase electrode, 192, 193
 - reaction enthalpy of, 11
- Glucose-6-phosphate, 198
- α -Glucosidase, 309
- β -Glucosidase, 320
 - determination of, 142
 - Glucosinolate, 192, 308
 - Glukometer, 103, 130, 131, 132, 190, 263, 293, 295–299, 306, 311, 316
- Glutamate, 158, 159, 222, 239, 243, 287, 296
- Glutamate decarboxylase, 159
- Glutamate dehydrogenase, 159
- Glutamate oxidase, 158, 296
- Glutamine, 232, 233, 239, 321
- Glycerokinase, 294
- Glycerol, 181, 321
- Glycerol ester hydrolase, 181
- Glycerophosphate oxidase, 294
- Glycolate, 149
- Glyoxylate, 149, 229
- Gonadotrophin-releasing hormone, 241
- Heavy metal ions, 320
- Hemoglobin sensor, 213–215
- Hepatitis B surface antigen, 276, 277
- Hexokinase, 11, 38, 88, 272, 294
 - antiinterference layer, 217
 - glucose oxidase electrode, 213
 - glucose-6-phosphate dehydrogenase electrode, 198, 214
- Hg^{2+} determination, 262, 263
- Histamine, 158
- Higher integrated systems, 230
- Horseradish peroxidase, 38, 90–92
 - catalase electrode, 214
 - electrochemical conversion, 33
 - glucose dehydrogenase electrode, 222
 - isocitrate dehydrogenase electrode, 152
 - as marker enzyme, 268, 271, 275, 276
- Hybrid sensor, 231
- Hydrazine, anodic oxidation of, 168
- Hydrogenase, 117, 156
 - electrochemical conversion of, 33
- Hydrogen peroxide determination
 - by electrochemical oxidation, 27
 - using a tissue-based sensor, 249
- Hydrolases, 38
- Hydroxybutyrate dehydrogenase, 149
- Hydroxybutyric acid, 149
- β -Hydroxysteroid dehydrogenase, 148
- Hypoxanthine, 210–212
- Immobilization, 50–53
 - effects of, 53–66
- Immuno field effect transistors, 283
- Immunoassay, 264
- Immunoglobulin, 48
 - determination of, 272, 276–278, 284–287
- Immunosensors, 275–284

- Immunostirrer, 272
- Inhibition of enzyme reactions, 46
- Inhibitors, 260–264
- Inosine, 210–212
- Insecticides, 320
- Insulin, 268, 276, 294
- Invertase, 183, 296, 319, 320
 - glucose oxidase electrode, 187–190, 217
 - Ion gate membrane, 30
- Ion selective electrode, 19–21
- Ion sensitive field effect transistor, 20, 21
- Isocitrate, 152, 153
- Isocitrate dehydrogenase, 152
- Isomerases, 38

- Katal, 45
- Kinetics of enzyme reactions, 41–45

- Laccase, 138, 230
 - antiinterference layer, 103, 218, 219
 - cytochrome b₂ electrode, 223–225
 - electrochemical conversion of, 33
- β -Lactamase, 11, 118, 176–180, 320
- Lactate dehydrogenase, 37, 126, 128, 133, 149, 222, 229, 230
 - characteristics, 126
 - cytochrome b₂ electrode, 222, 227
 - determination of, 131, 132, 253, 296, 309
 - lactate monooxygenase electrode, 199–204
 - lactate monooxygenase-pyruvate kinase electrode, 201–204
 - lactate oxidase electrode, 81, 222–226, 228
 - oxalate oxidase electrode, 149
 - reaction enthalpy, 11
- Lactate determination, 126–135, 304–306, 316
 - with automatic analyzers, 130, 295, 304–306, 317
 - with enzyme thermistors, 127, 222, 224, 294
 - with an erythrocyte sensor, 127
 - with the Glukometer, 130, 296, 306
 - with an organelle sensor, 233
 - using substrate recycling, 81, 222–226
- Lactate monooxygenase, 37, 127, 130, 131, 294, 296, 309
 - lactate dehydrogenase electrode, 199–204
 - malate dehydrogenase electrode, 203
- Lactate oxidase, 131, 296, 309
 - lactate dehydrogenase electrode, 81, 222–226, 228
 - thermistor, 222, 224
- Lactate/pyruvate ratio, 200
- Lactose determination, 192, 295, 296, 320
 - in milk, 317
- Langmuir-Blodgett film, 252
- Lectin, 254
- Lidocaine, 267, 268
- Ligases, 39
- Lineweaver-Burk plot, 43, 44
 - electrochemical, 63
- Lipase, 182
- Lipid membrane biosensors, 252
- Lipoprotein lipase, 294
- Liposomes, 270, 278
- Logical operations in biosensors, 324
- Luciferase, 16
- Luminescence, 13, 16
- Lyases, 38
- Lysine, 158, 295
- Lysozyme, 35

- Malate, 203, 223
- Malate dehydrogenase-lactate monooxygenase electrode, 203
- Maltase, 187
 - glucose oxidase electrode, 191
- Maltitol, 261
- Maltose, 76, 190–192, 218, 296
- Mannan, 255
- Maximal rate of an enzyme reaction (v_{\max}), 41–45
- Meat freshness sensor, 143, 210–212
- Mediators, 28–32
- Metabolism sensors, definition, 8, 9
- Methanol, 136–138
- Methanol oxidase, 137
- Methylviologen, 28

- Michaelis constant (K_M), 42–45
Michaelis Menten equation, 42
Microbial sensors
 overview, 238–241, 245
 for characterization of
 microorganisms, 246–248
Microelectrochemical device, 23
Microelectronics, 323
Microsomal electrode, 234–236
Mitochondrial sensor, 4, 232, 233
Molecular recognition element, 4
Monoamine oxidase, 143
Morphine, 267, 287
Mutagenicity test, 244–246
Mutarotase, 39, 187, 296
 Myrosinase, 192

NAD⁺, 40
 chip, 253
 determination of
 using a competition sensor, 212–214
 using substrate recycling, 222, 230
NADH determination
 by anodic oxidation, 28, 29
 with optical sensors, 15, 16
 with organelle sensors, 4, 232–235
 using substrate recycling, 222, 229, 230
NADP⁺, 40
 determination of, 198, 199
NADPH determination
 by anodic oxidation, 28, 29
 with an organelle sensor, 232–235
Neurons, 287, 288
Neutrophil leucocytes, 289
Nicotinic acetylcholine receptor, 287, 288
Nitrate, 154, 155
Nitrite, 154, 155
Nitrophenylphosphate, 15
N-methylphenazinium, 28
Nucleoside phosphorylase, 210
5'Nucleotidase, 210

Open tubular reactors, 89
Optoelectronic sensors, 8, 9, 13–18, 106, 107, 138, 161, 171, 177, 178, 254, 284–287, 292

Organelles, 4, 8
Organelle sensors, 232–236
Organic metal electrodes, 31, 115
Oxalate, 153, 249, 294
Oxalate oxidase, 153, 294
 -lactate dehydrogenase electrode, 149
Oxaloacetate, 154, 203, 223
Oxaloacetate decarboxylase–pyruvate oxidase electrode, 204, 310
Oxidoreductases, 37
Oxygen electrode, 3, 25, 26, 29, 30
Oxygen limitation of glucose sensors, 95–98
Oxygen-stabilized enzyme electrode, 29, 318

Palladium MOSFET, 22, 23
Parallel coupling of enzyme reactions, 185
Parathion, 284
Pb²⁺ determination, 262
Penicillin, 16, 118, 176–181, 293, 320
Penicillin amidase, 176
Penicillinase, 176, 177
Pesticides, 262
pH dependence of enzyme electrodes, 64
pH electrode, 19
Phenol, 138–143, 213, 240, 249, 320
 determination in chloroform, 142
Phenol hydroxylase, 140, 141
Phenytoin, 269
Phosphate, 249, 250
Piezoelectric crystal, 10, 18, 259
Piezoelectric immunosensors, 18, 259, 283, 284
Polyamine oxidase, 143
Potato tissue–glucose oxidase electrode, 250
Potentiometric electrodes, 18–24
Progutrin, 192
Proinsulin, 273, 275
Prosthetic group, 8, 39
 determination of, 259
Protein determination, 158
Proteolytic enzyme field effect transistor, 159

- Pyruvate, 135, 199, 200, 222, 227, 249, 296, 316
Pyruvate kinase, 201, 296, 309, 310
 -hexokinase electrode, 223, 227
Pyruvate oxidase, 127, 135
- Reaction-transport coupling, 53-56
Receptor, 1-4, 8, 9, 49, 50, 287-289
Receptrode, 5, 253, 287-289
Redox mediators, 28-31
Reflectometry, 13, 17
Salicylate, 153
Sarcosine oxidase, 60, 209
Sensors using higher integrated systems, 230-252
Sequential coupling of enzyme reactions, 184
Silicon-on-sapphire sensor, 122-124
- Single bead string reactor, 89
Sinigrin, 192
Sitosterol, 148
Starch, 193-195
Sterilizable enzyme electrode, 179
Substrate recycling, 185, 203, 220-230, 324
 modeling of, 78-80
 with only one enzyme, 229
Succinate, 232, 239
Sucrose, 183, 187-190, 194, 216, 217, 295, 296, 316, 320
Sulphate, 241, 261
Sulphide, 241
Sulphite, 155, 233
Sulphite oxidase, 155
Synzyme, 4
 electrode, 154
- Temperature dependence
 of enzyme electrodes, 64
 of enzyme reactions, 46
Test strips, 3, 292, 293
Tetracyano-p-quinodimethane, 28, 30, 31
Tetrathiafulvalene, 28
- Theophylline, 268, 271
Thermistor, 10-13
Thermometric enzyme immunoassay, 273-275
Thyroxine, 267, 268
Tissue-based sensors, 248-251
Transaminases, 201-204, 310
Transducers, 8-34
Transferases, 38
Triglycerides, 181, 294
Trypsin, 11
Turnover number, 45
Two-dimensional enzyme electrode, 97
Tyrosinase, 142
Tyrosine, 158, 249
- Urea determination, 159-174, 303, 304
 with amperometric electrodes, 167-170, 304
 with antimony electrodes, 166
 with conductometric sensors, 170
 with enzyme reactors, 161
 with enzyme thermistors, 294
 in dialyzate, 303, 313
 with field effect transistors, 122-124, 171-174
 with the Glukometer, 296
 with a hybrid sensor, 167, 241-243
 with optical sensors, 15, 161, 162, 171, 293
 in serum, 169, 303
 with tissue-based sensors, 249
Urease, 60, 263, 264, 294, 296, 320
 characteristics, 159
 inhibition of, 263
 reaction enthalpy of, 11
Uric acid, 149, 294, 306
Uricase, 11, 149, 196
 -horseradish peroxidase electrode, 150
- Xanthine oxidase, 210
 electrochemical conversion of, 33
Zn²⁺ determination, 262

This Page Intentionally Left Blank